การศึกษาทางสรีรวิทยาถึงผลของสารสกัดจากแตงโม (*Citrullus lanatus*) ต่อการหดตัวของมดลูกหนู

นายพักพล มุ่งลือ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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PHYSIOLOGICAL INVESTIGATION OF THE EFFECTS OF WATERMELON (*CITRULLUS LANATUS*) EXTRACTS ON RAT UTERINE CONTRACTION

Phukphon Munglue

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Environmental Biology

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Academic Year 2011

PHYSIOLOGICAL INVESTIGATION OF THE EFFECTS OF WATERMELON (*CITRULLUS LANATUS*) EXTRACTS ON RAT UTERINE CONTRACTION

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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พักพล มุ่งลือ : การศึกษาทางสรีรวิทยาถึงผลของสารสกัดจากแตงโม (*Citrullus lanatus*) ต่อการหดตัวของมดลูกหนู (PHYSIOLOGICAL INVESTIGATION OF THE EFFECTS OF WATERMELON (*CITRULLUS LANATUS*) EXTRACTS ON RAT UTERINE CONTRACTION) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ สัตวแพทย์หญิง ดร.ศจีรา คุปพิทยานันท์, 227 หน้า.

แตงโม (*Citrullus lanatus*) มีแอล-ซิทูไลน์และแอล-อาร์จินีนในปริมาณสูง สารดังกล่าวมีความสำคัญในการสร้างไนตริคออกไซด์ที่มีฤทธิ์คลายหลอดเลือด แตงโมมีฤทธิ์ทางชีวภาพแต่ยังไม่มีผลการศึกษาถึงฤทธิ์ทางสรีรวิทยาต่อการหดตัวของกล้ามเนื้อมดลูก ดังนั้น การศึกษานี้มีวัตถุประสงค์เพื่อศึกษา 1) ผลของสารสกัดจากแตงโมต่อการหดตัวของมดลูกหนู 2) กลไกออกฤทธิ์ของสารสกัด และ 3) ฤทธิ์ของสารสกัดเป็นผลของแอล-ซิทูไลน์และ/หรือแอล-อาร์จินีน เนื้อและเปลือกแตงโมสกัดด้วยเอทิลแอลกอฮอล์ การตอบสนองของการหดตัวต่อสารต่าง ๆ ของมัดกล้ามเนื้อมดลูกถูกบันทึกด้วยอุปกรณ์ทดสอบการหดตัวของมัดกล้ามเนื้อ ผลการศึกษาพบว่า ความเข้มข้นของสารสกัดจากเนื้อที่ 6 มก./มล. เปลือก 5 มก./มล. แอล-ซิทูไลน์ 64 ไมโครโมลาร์ และแอล-อาร์จินีน 104 ไมโครโมลาร์ สามารถยับยั้งการหดตัวโดยธรรมชาติได้ร้อยละ 50 สารสกัดจากแตงโม แอล-ซิทูไลน์และแอล-อาร์จินีนมีฤทธิ์ยับยั้งการหดตัวที่ถูกเหนี่ยวนำด้วยพรอสตาแกลนดินเอฟ 2 แอลฟา ออกซิโทซินและสารละลายโพแทสเซียมคลอไรด์ ในภาวะที่ปราศจากแคลเซียมนอกเซลล์พบว่าสารสกัดจากแตงโม แอล-ซิทูไลน์และแอล-อาร์จินีนมีฤทธิ์ยับยั้งการหดตัวที่ถูกเหนี่ยวนำด้วยพรอสตาแกลนดินเอฟ 2 แอลฟาและออกซิโทซินและยังยับยั้งการหดตัวที่ถูกเหนี่ยวนำด้วยการเพิ่มปริมาณแคลเซียมนอกเซลล์ได้บางส่วน ในขณะเดียวกันสารสกัดจากแตงโม แอล-ซิทูไลน์และแอล-อาร์จินีนมีฤทธิ์ยับยั้งการหดตัวแบบโทนิคที่ถูกเหนี่ยวนำด้วยออกซิโทซินในสารละลายโพแทสเซียมคลอไรด์ สารสกัดแตงโมสามารถออกฤทธิ์ร่วมกับแอล-ซิทูไลน์ในการยับยั้งการหดตัวโดยธรรมชาติและการหดตัวที่เหนี่ยวนำด้วยพรอสตาแกลนดินเอฟ 2 แอลฟา ออกซิโทซินและสารละลายโพแทสเซียมคลอไรด์ การค้นพบครั้งนี้ชี้ให้เห็นว่ากลไกออกฤทธิ์ของสารสกัดจากแตงโม แอล-ซิทูไลน์และแอล-อาร์จินีนผ่านทั้งวิถีที่เกี่ยวข้องและไม่เกี่ยวข้องกับแคลเซียม ในขณะเดียวกันฤทธิ์ยับยั้งการหดตัวของสารสกัดจากแตงโมและแอล-ซิทูไลน์ยังสามารถผ่านวิถี

ของไนตริคออกไซด์-ไซคลิคจีเอ็มพีและการกระตุ้นประตูโพแทสเซียมได้เช่นเดียวกัน สรุปได้ว่าแตงโมมีฤทธิ์ยับยั้งการหดตัวของมดลูกหนู

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ลายมือชื่อนักศึกษา_____
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PHUKPHON MUNGLUE : PHYSIOLOGICAL INVESTIGATION OF THE EFFECTS OF WATERMELON (*CITRULLUS LANATUS*) EXTRACTS ON RAT UTERINE CONTRACTION. THESIS ADVISOR : ASSOC. PROF. SAJEERA KUPITTAYANANT, Ph.D. (DVM), 227 PP.

CITRULLUS LANATUS/ WATERMELON/ UTERUS/ CONTRACTION/ L-CITRULLINE/ NITRIC OXIDE

Watermelon (*Citrullus lanatus*) is rich in L-citrulline and L-arginine; the contents that play an important role in the production of the potent vasodilator, nitric oxide (NO). The plant has been studied for its biological activities, but not for its physiological activities in uterine smooth muscle. Thus, this study aimed; 1) to investigate the effects of watermelon extracts on rat uterine contractions; 2) to investigate the mechanisms whereby the extracts exerted their effects; and 3) to verify whether the effects of the extracts were due to their major constituents, L-citrulline and/or L-arginine. Watermelon flesh and rind were ethanolic extracted. The contractile responses of each agent were recorded isometrically with a force transducer. The results showed that the EC_{50} values of flesh, rind, L-citrulline, and L-arginine were 6 mg/mL, 5 mg/mL, 64 μM, and 104 μM, respectively. They suppressed spontaneous contraction and the contractions induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), oxytocin and potassium chloride solution (KCl). They also reduced $PGF_{2\alpha}$- and oxytocin-induced uterine force in the absence of external calcium (Ca^{2+}) and partially inhibited contraction induced by increasing external Ca^{2+}. In addition, they caused a marked decrease in tonic contractions produced by oxytocin-induced uterine

contraction in the presence of KCl. The combinations of watermelon extracts and L-citrulline elicited an additive effect on spontaneous contraction and uterine contractions induced by $PGF_{2\alpha}$, oxytocin, and KCl. These findings indicated that the mechanisms of action of watermelon extracts, L-citrulline, and L-arginine were via Ca^{2+}-dependent and Ca^{2+}-independent regulation of smooth muscle contraction pathways. In addition, the tocolytic effects of watermelon extracts and L-citrulline were also via NO-cGMP pathway as well as via the activation of calcium-activated potassium channels. In conclusion, the study clearly showed that watermelon has a potent tocolytic effect on rat uterine contraction.

School of Biology

Academic Year 2011

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CHAPTER I

INTRODUCTION

Scientists have long been investigating the underlying mechanisms of uterine contractility. However, they have generally focused on basic phenomena which modulate the function of the muscle at each stage of reproductive periods. Moreover, the main points of the investigations have been dedicated to the action of agents which either inhibit (tocolytic agents) or stimulate (uterotonic agents) uterine contractions. Current uses of interventional treatments lack potency and/or selectivity and may do harm to the uterus. Thus, new approaches to tocolytic or uterotonic are required. Medicinal plants are used as the starting materials for pharmaceutical development worldwide. Interestingly, they are safe and have less side-effects. Therefore, the work presented in this thesis was carried out to search for a novel plant species for use as a tocolytic or uterotonic.

1.1 Uterus and Its Functions

The *uterus (womb)* is a complex organ that is specifically adapted for the reproductive process. It has 3 compartments: the horns, body and cervix. All of these have a different size and shape according to species (Constantinescu, 2007). The uterus is supported by cords of the uterine ligaments, located in the pelvis immediately dorsal to the urinary bladder and ventral to the rectum. One end is

connected on both sites to the fallopian tube; the other, the cervix, opened to the vagina. In most animals, it is well established that the uterus has 2 horns, a body and a cervix, and it is known as the *uterus bicornis* (Constantinescu, 2007). In primates, including humans, the uterus has only one segment and the cervix, which is classified as the *uterus simplex*. In the rabbit, for example, the horns, body and cervix are paired; this type of uterus is determined as the *uterus duplex*. Moreover, in cattle, sheep and houses, the uterus is of the *uterus bipartitus*.

The uterus plays a functional role in the reproductive process: 1) sperm transport from the site of ejaculation to the site of fertilization in the oviduct; 2) initiation of implantation, pregnancy and parturition; and 3) regulation of the function of the corpus luteum (Hafez and Hafez, 2000).

Knowledge of various types of cells, their functions, interactions, and relationships is essential for a complete understanding of uterine physiology. Changes which occur in uterine muscle during pregnancy, menstrual period, and estrus cycle are important physiological considerations. There is evidence that changes in the levels of several hormones, including steroid hormones, oxytocin, and prostaglandins play a crucial role in modulating the uterine functions in each stage throughout the female reproductive life (Thomson and Norman, 2005).

1.1.1 Anatomy of the Uterus

On a histological basis, the uterus has traditionally been divided into the endometrium and the myometrium. The endometrium is a mucosal layer constituted by specialized glandular epithelium and a highly cellular stroma which respond with

cyclical change of growth, differentiation and shedding in reply to ovarian hormones throughout the reproductive life of the female. The myometrium is a muscular tunic which comprises of a three-layer smooth muscle: a longitudinal outer layer and circular and oblique inner layer. It surrounds the endometrial lining of the uterine cavity and forms the major component of the uterine volume (Fusi, Cloke and Brosens, 2006).

1.1.2 Contractile Apparatus

Myometrial smooth muscle cells contain the contractile apparatus that respond to calcium (Ca^{2+}) oscillations and utilize the chemical energy of adenosine triphosphate (ATP) during shortening or tension development. It is widely accepted that the major contractile proteins are myosin, actin, tropomyosin, and minor components of contractile apparatus include the proteins that are involved in the Ca^{2+}-dependent regulatory mechanism (Horowitz, Menice, Laporte and Morgan, 1996).

The Thick Filaments

Myosin (thick filaments) is the main element of muscle contraction. It is a structural protein which interacts with the other major proteins, including actin. Myosin is also an enzyme that converses the chemical energy of ATP into force generation. In smooth muscle cells, such as the myometrium, the myosin molecule is a hexamer molecule composed of two heavy chains (MHCs) and two light chains (MLCs) of 20 kDa (MLC_{20}) and 17 kDa (MLC_{17}). The native polymer of myosin contains three regions. The tail domain is configured as α-helical coiled tail regions of

myosin (rods) forming the thick filament with the globular head regions protruding to form cross-bridges. The head domain consists of the globular N-terminal end of the MHC that protrudes from the filament. It was found that the head domain contains the actin-binding region and the ATP hydrolysis site that serves as a source of the energy for force development (Adelstein and Sellers, 1995). The intermediate (neck) domain is the region that creates the angle between the head and the tail. It serves as the non-covalent binding sites of the MLCs. In uterine smooth muscle, MLC_{20}, known as regulatory light chain, is essential for regulating muscle contraction. The MLC_{17} is called the essential light chain and its function is unknown. It has been reported that phosphorylation of Serine 19 on MLC_{20} results in a conformational change that increases the angle in the neck domain of the MHC, causing the actin thin filament to slide along the myosin thick filament (Adelstein and Sellers, 1995). This phosphorylation is mediated by myosin light chain kinase (MLCK), which is predominantly modulated by the intracellular Ca^{2+} ion ($[Ca^{2+}]_i$) (Adelstein and Sellers, 1995; Horowitz et al., 1996; Word and Kamm, 1997).

The Thin Filaments

Native thin filament isolated from smooth muscle tissues contains actin and tropomyosin. In the presence of Ca^{2+}, thin filament serves as the modulatory protein in smooth muscle. It was found that actin filaments slide along the myosin thick filament to shorten the cell during a contraction (Lehman, Vibert, Craig and Bárány, 1995). This process has three basic requirements: 1) a force is required to move the actin filaments; 2) the force must be transmitted along the actin filament from the

longitudinal poles of the cell towards the cell center; and 3) the actin must be attached to the cytoskeleton of the myocyte (Lehman et al., 1995).

Calmodulin

Calmodulin (CaM), a Ca^{2+}-binding protein, mediates many of the regulatory effects of Ca^{2+}, including the contractile state of smooth muscle. The prominent function of CaM in smooth muscle is to activate cross-bridge cycling and force development in response to a $[Ca^{2+}]_i$ oscillation. This process includes 1) the activation of MLCK and 2) phosphorylation of myosin. CaM is often referred to as EF-hand Ca^{2+}-binding proteins. Crystallographic study indicated that CaM has four EF-hands consistent with the presence of four Ca^{2+} binding sites (Means, 2004). It was revealed that the binding of Ca^{2+} to CaM induces a marked conformational change and leads to the interaction with target proteins (Walsh, 1994). It has been reported that binding of Ca^{2+} to three or all four sites is required for activation of MLCK (Walsh, 1994; Word and Kamm, 1997).

Myosin Light Chain Kinase

Myosin light chain kinase (MLCK) is Ca^{2+}-CaM regulated serine or threonine protein kinase that catalyzes the phosphorylation of MLC_{20}. MLCK is activated by the binding of 1 mol Ca^{2+}-CaM to 1 mol kinase. It was found that MLCK contains a catalytic core that is highly homologous to other protein kinase (Walsh, 1994). The catalytic core of MLCK contains two lobes with the smaller lobe binding MgATP. The phosphorylation occurs at a cleft between the two lobes, with the larger lobe

serving as the binding site for protein substrates. By using tryptic cleavage enzyme method, it was indicated that MLCK composes of an inhibitory region and a CaM-binding region (Blumenthal and Stull, 1980). It was suggested that the inhibitory region mimics the light chain substrate, and is a pseudosubstrate prototype that binds to the active site of the kinase in the absence of CaM (Blumenthal and Stull, 1980). It appears that autoinhibition is associated with the folding of a C-terminal extension of the kinase back onto the catalytic core (Kamm and Stull, 2001). Binding of Ca^{2+}-CaM to MLCK induces a conformational change, leading to dissociation of the pseudosubstrate domain from the substrate binding site and production of force (Stull et al., 1995).

Myosin Light Chain Phosphatase

Myosin light chain phosphatase (MLCP), a serine and threonine protein phosphatase, requires to reverse the activation of smooth muscle myosin by MLC_{20} phosphorylation. It is well known that decrease of $[Ca^{2+}]_i$ inactivates Ca^{2+}-dependent MLCK, shifting the balance of kinase and phosphatase activities resulting in dephosphorylation of MLC and smooth muscle relaxation (Word and Kamm, 1997). Inhibition of MLCP activity by some agonists is the main mechanism of Ca^{2+}-sensitization. It was indicated that inhibition of the phosphatase increases MLC_{20} phosphorylation and force production (Erdödi, Ito and Hartshorne, 1995). Arachidonic acid is found to be a Ca^{2+}-sensitizing agent (Gong et al., 1992). It increases both MLC_{20} phosphorylation and contraction at constant Ca^{2+} by inhibiting MLC_{20} dephosphorylation (Gong et al., 1992). Ca^{2+}-desensitization phenomena

mediated by cGMP and cGMP-dependent protein kinase (PKG) may be due to upregulation of MLCP (Somlyo and Somlyo, 1994; 1998).

1.1.3 Uterine Contractile Activity

Electromechanical and Pharmacological Coupling

It is well established that modulation of contractile force by changing in membrane potential is referred to as electromechanical coupling (Itoh and Kuriyama, 1994; Somlyo and Somlyo, 1994; 1998). This type of modulation is primary associated with a change of $[Ca^{2+}]_i$. Depolarization of smooth muscle (either by potassium chloride solution (KCl) or agonists) stimulates several types of Ca^{2+} channels and generates contraction. The relationship between changes in membrane potential and force development is based on stimuli. It is indicated that contractile agonists must induce contraction with less depolarization than that required by KCl (Chen and Rembold, 1995; Rembold, 1995). However, some agonists can increase Ca^{2+} inward current at a constant membrane potential in smooth muscle cells. These findings indicated that pharmacomechanical coupling may represent by agonist induced increase in $[Ca^{2+}]_i$ beyond that expected by the level of depolarization (Chen and Rembold, 1995; Rembold, 1995). The mechanisms underlying of this circumstance include: 1) an agonist-dependent increase in Ca^{2+} influx, resulting in higher $[Ca^{2+}]_i$; 2) an agonist-dependent increase in the Ca^{2+}sensitivity of myosin phosphorylation; and 3) other unknown processes (Rembold, 1995).

It is well known that regulation of force independent of changes in membrane potential is referred to as pharmacomechanical coupling (Somlyo and Somlyo, 1994;

1998). This type of modulation is involved in either changes in $[Ca^{2+}]_i$ or changes in the cellular response to $[Ca^{2+}]_i$ independent of changes in membrane potential (Chen and Rembold, 1995; Rembold, 1995). Modulation of myosin phosphorylation independent of changes in $[Ca^{2+}]_i$ is another pathway of pharmacomechanical coupling (Somlyo and Somlyo, 1994; 1998; Rembold, 1995). Apparent alterations in the $[Ca^{2+}]_i$ sensitivity of force may be caused by: 1) changes in the time course of $[Ca^{2+}]_i$ and myosin phosphorylation (Rembold, 1995); 2) changes in the $[Ca^{2+}]_i$ sensitivity of phosphorylation (Somlyo and Somlyo, 1994); and/or 3) changes in the relationship between myosin phosphorylation and force (Rembold, 1995).

Mechanisms of Uterine Contraction

It is well known that the contraction of uterine smooth muscle is due to Ca^{2+} binding to CaM activating MLCK, leading to the phosphorylation and subsequent cross-bridge cycling (Somlyo and Somlyo, 1994). This Ca^{2+} is from 2 main sources to operate this incidence: 1) release from the sarcoplasmic reticulum (SR) via inositol (1,4,5)-tris-phosphate (IP_3) or ryanodine receptor (RyR)/Ca release channels; and 2) from extracellular source via voltage-gated L-type Ca^{2+} channels or receptor-operated Ca^{2+} channels (Kupittayanant, Luckas and Wray, 2002). In addition, another major source of Ca^{2+} for contraction is from the resulting of action potentials, depolarization, and then consequent opening of L-type Ca^{2+} channels (Matthew, Shmygol and Wray 2004). Wray, Kupittayanant, Shmygol, Smith and Burdyga (2001) indicated that nifedipine, a blocker of L-type Ca^{2+} channels, prevents the inward current and there is no rise in Ca^{2+}, leading to abolition of both the

contractions and transients of the uterus. Interestingly, the uterine contraction is also failed to occur if MLCK is inhibited by wortmannin, a selective inhibitor of MLCK (Longbottom, Lukas, Kupittayanant, Badrick, Shmygol and Wray, 2000). This finding suggested that Ca^{2+}-calmodulin-MLCK pathway plays a crucial role in regulating the uterine contractility (Wray, 2007), as shown in Figure 1.1.

However, under certain condition, $[Ca^{2+}]_i$ is not induced to phosphorylate MLC and contraction. In the "tonic force" condition, the relation of force and MLC phosphorylation levels can be dissociated (Somlyo and Somlyo, 1994). The another pathway that free from Ca^{2+} activities to control smooth muscle contraction has been established as the non-Ca^{2+}-CaM-MLCK pathways (Somlyo and Somlyo, 1994). Protein kinase C and Rho-associated kinase, for example, are the well-known mechanisms to regulate the uterine contractility (Somlyo and Somlyo, 1994).

Moreover, caldesmon and calponin, thin filament-associated proteins, can be activated by phosphorylation of mitogen-activated protein kinase and/or other kinases to regulate myosin MgATPase activity (Somlyo and Somlyo, 1994).

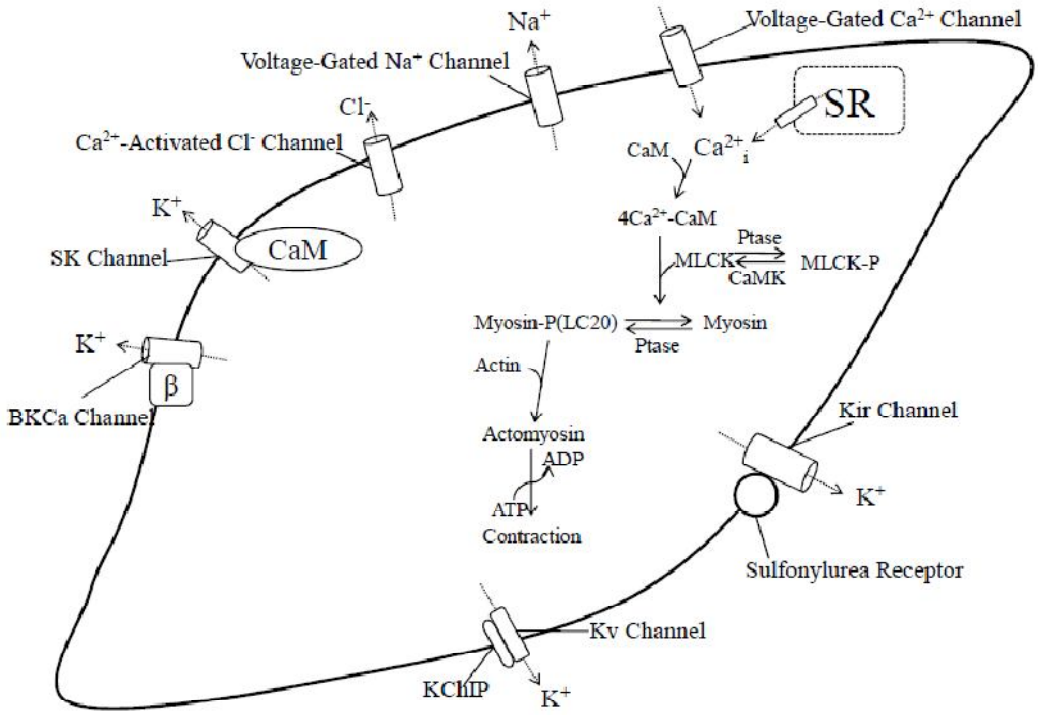

Figure 1.1 A schematic diagram of a myometrial smooth muscle cell showing a variety of ion channels that are involved in regulation of membrane potential and cell excitability. Uterine contraction occurs as the phosphorylation of myosin light chain (MLC_{20}) by myosin light chain kinase (MLCK). For more details see the text. ADP = adenosine diphosphate; ATP = adenosine triphosphate; K_{ATP} = the ATP-sensitive K^+ channel; Kv = the *Shaker*-like voltage-gated potassium channels; SK channel = the small-conductance calcium-sensitive potassium channels; BKCa channel = the large-conductance calcium- and voltage-sensitive K^+ channel; SR = sarcoplasmic reticulum; Ptase = protein phosphatase; MLCK-P = phosphorylated myosin light chain kinase; CamK = calcium calmodulin-dependent protein kinase; KChIP = Kv channel interacting protein; CaM = calmodulin. The schematic diagram is adapted from Brainard, Korovkina and England (2007).

Mechanisms of Uterine Relaxation

Contraction of the myometrial cells is terminated by: 1) dephosphorylation of MLC via MLCP; 2) $[Ca^{2+}]_i$ returns to resting levels via a sarcolemmal Ca^{2+} pump or Na^{+}/Ca^{2+} exchange; and 3) removing Ca^{2+} from the cytoplasm to the SR via sarcoplasmic reticulum Ca^{2+}- ATPase. All these results in dissociation of Ca^{2+} from CaM and inactivation of MLCK lead to uterine relaxation (Wray, 1993; 2007).

In another pathway, nitric oxide (NO) may cause smooth muscle relaxation via: 1) the stimulation of calcium-dependent potassium (K_{Ca}) channels; 2) the activation of guanylate cyclase; and 3) the stimulation of ADP ribosylation (Lincoln, Cornwell, Komallavilas, Macmillan-Crow and Boerth, 1995). The activation of guanylate cyclase is probably the mechanism of NO to relax uterine smooth muscle (Figure 1.2). As described by Yallampalli, Garfield and Byam-Smith (1993), methylene blue, an inhibitor of guanylate cyclase, has been used to inhibit smooth muscle relaxation produced by NO. However, this role for guanylate cyclase seems to be less sensitive to the relaxant effects of cyclic GMP (cGMP) than vascular smooth muscle (Word, Casey, Kamm and Stull, 1991). It was indicated that inhibition of K_{Ca} channels by using 1 mM tetraethylammonium, a concentration that blocks uterine smooth muscle K_{Ca} channels when applied to the extracellular side, can cause smooth muscle contraction (Anwer, Toro, Oberti, Stefani and Sanborn, 1992). Lastly, Brüne and Lapetina (1990) reported that NO causes adenosine diphosphate (ADP) ribosylation in platelets, which ultimately may inhibit glycolysis (Kots, Skurat, Sergienko, Bulgarina and Severin, 1992).

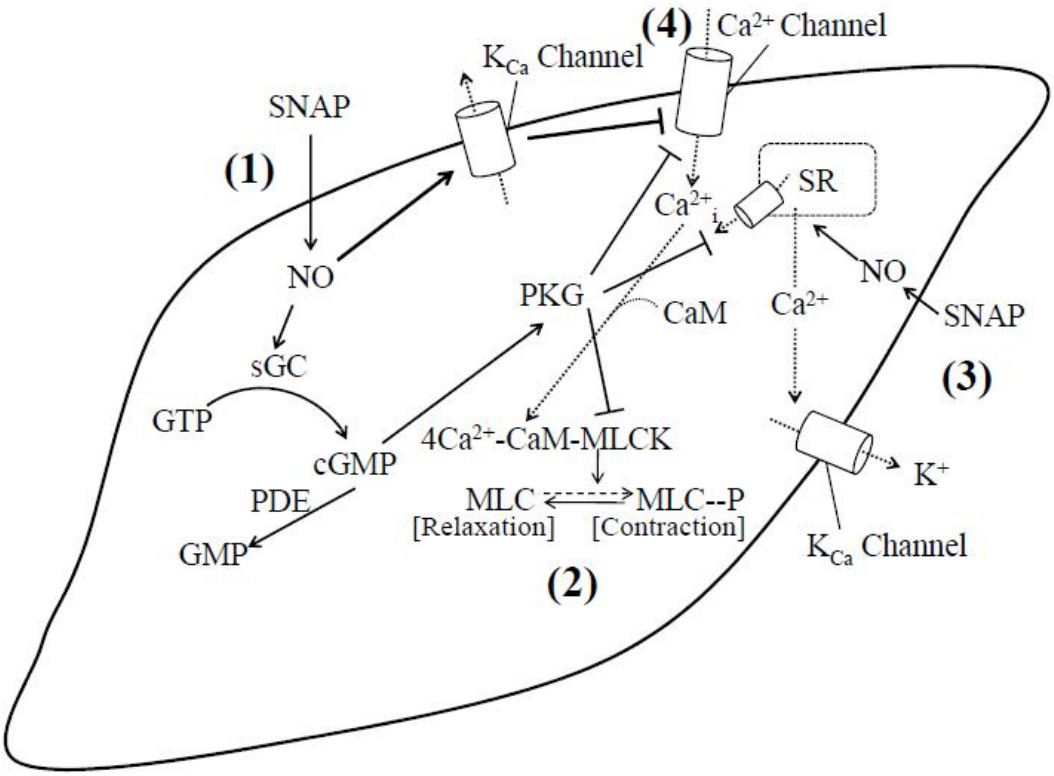

Figure 1.2 A schematic diagram of a myometrial smooth muscle cell relaxation mediated by nitric oxide (NO). **(1)** *S*-Nitroso-*N*-acetylpenicillamine (SNAP), a nitric oxide donor, generates NO to activate soluble guanylate cyclase (sGC) and the accumulation of cGMP. **(2)** Activation of protein kinase G (PKG) by cGMP results in the activation of the kinase and the subsequent phosphorylation of substrate proteins in the cell. The interaction of PKG and the myosin phosphatase that leads to its activation and ability to dephosphorylate the myosin regulatory light chains (not shown) must be questioned in myometrium because elevation of cGMP does not relax the tissue. This interaction between PKG and myosin phosphatase is known to be critical in other smooth muscles because inhibition of the myosin phosphatase leads to increased force for a given concentration of Ca^{2+} (Ca^{2+}-sensitization). **(3)** SNAP may be caused the uterine relaxation by generating Ca^{2+} efflux from SR, resulting in the activation of K_{Ca} channel. The extrusion of K^+ leads to hyperpolarization of the cell

membrane. **(4)** Hyperpolarization of the membrane inhibits the influx of Ca^{2+} via Ca^{2+}channel, leading to decreasing intracellular Ca^{2+} concentration and relaxing the muscle. The schematic diagram is adapted from Buxton (2004).

1.2 Calcium Signaling and Uterine Contraction

As mentioned above, there are at least 2 pathways for the contraction of uterine smooth muscle: 1) Ca^{2+}-CaM-MLCK pathways and 2) non-Ca^{2+}-CaM-MLCK pathways. However, Wray et al. (2001) and Wray (2007) have concluded that the Ca^{2+}-CaM-MLCK pathway is the major mechanism to maintain uterine force in normal physiological conditions.

1.2.1 Ca^{2+}-CaM-MLCK Pathways

A variety of hormones, neurotransmitters, and pharmacological agents can be used to modulate the contractile activity of uterine smooth muscle cells (Wray, 1993). Stimuli induce smooth muscle contraction by four mechanisms (Rembold, 1995). 1) Both contractile agonists and KCl depolarize smooth muscle and then increase Ca^{2+} influx though voltage-dependent Ca^{2+} channels. 2) Agonists induce Ca^{2+} release from the SR. 3) Agonists activate both voltage-dependent and voltage-independent Ca^{2+} channels, leading to increase Ca^{2+} influx that is not associated with depolarization. 4) Agonists can increase Ca^{2+} sensitivity. This means force production can be enhanced by agonists in a given $[Ca^{2+}]_i$ condition (Rembold, 1995). Immediately after elevation of $[Ca^{2+}]_i$, homeostatic mechanisms mediates to restore the resting condition by active removal of the excess Ca^{2+} from intracellular through energy-driven extrusion

mechanisms (pumps) and channels present in the plasmamembrane and the membranes of the SR (Riemer and Heymann, 1998).

L-Type Ca^{2+} Channels

In smooth muscle cells, two types of Ca^{2+} channels have been described (Riemer and Heymann, 1998; Word and Kamm, 1997); an L-type and a T-type Ca^{2+} channels. The L-type Ca^{2+} channel inactivates slowly, is permeant more to Ba^{2+} than to Ca^{2+}, and is highly sensitive to dihydropyridine Ca^{2+} channel blockers or agonists. The T-type Ca^{2+} channel inactivates rapidly, is equally permeant to Ca^{2+} and Ba^{2+}, and is less sensitive to dihydropyrinine Ca^{2+} channel blockers. L-type Ca^{2+} channel serves as the main site of Ca^{2+} entry into the smooth muscle cell and the primary source of action potential (Word and Kamm, 1997). It is well established that the activation of L-type Ca^{2+} channel and Ca^{2+} entry will occur when uterine myocyte membrane potential is depolarized to -40 mV (Parkington and Coleman, 1990; Wray, 1993). The increased $[Ca^{2+}]_i$ activates K^+ channels, permitting K^+ efflux and restoration of membrane potential (Hofmann and Krugbauer, 1995; Riemer and Heymann, 1998).

Modulation of Ca^{2+} channels unrelated to membrane potential has been investigated by several investigators (Hofmann and Krugbauer, 1995; Riemer and Heymann, 1998). The phosphorylation of Ca^{2+} channels by protein kinases has been reported (Hofmann and Krugbauer, 1995). Studies on cultured rat aortic cells indicated that cAMP may reduce the Ca^{2+} inward current produced from voltage-gated Ca^{2+} channels. The prominent mechanism of cAMP-dependent protein kinase (PKA)

and PKG-mediated phosphorylation of voltage-gated Ca^{2+} is not well understood. It was indicated that the α1 and β_{2a} subunits of voltage-gated Ca^{2+} channel are the phosphorylation sites of PKA and the α1c subunit serves as the phosphorylation site of PKG (Hofmann and Klugbauer, 1995).

Calcium Release from SR

Agonists interact with a specific G-protein coupled receptor (GPCR) in the myocyte plasmamembrane resulting in activation of a trimeric G-protein containing a $G_{\alpha q}$ or $G_{\alpha 11}$ subunit. Activation of this subunit in the uterine myocyte stimulates a specific phospholipase C to hydrolyze PIP_2 into IP_3 and DAG (Phillippe and Chien, 1998). In smooth muscles, the physiological agent that induces Ca^{2+} release for contraction upon agonist stimulation is IP_3. IP_3 interacts with a specific receptor (IP_3R) to induce release of Ca^{2+} from the SR and subsequent rise in $[Ca^{2+}]_i$ (Phillippe and Chien, 1998). It has been reported that emptying of the SR Ca^{2+} stores increases tone in myometrium (Kupittayanant et al., 2002). In addition, emptying of the SR Ca^{2+} stores in these same experiments had little if any effect on the cytosolic Ca^{2+} concentrations achieved or the force generated following oxytocin stimulation (Kupittayanant et al., 2002). This finding indicates that the agonist-stimulated, IP_3-mediated release of Ca^{2+} is less important than the Ca^{2+} entry from the extracellular space through L-type Ca^{2+} channels (Kupittayanant et al., 2002). It is suggested that the SR may serve as a sink of Ca^{2+} clearance from the cytosol after action potential (Shmigol, Eisner and Wray, 1999; Taggart and Wray, 1998).

1.2.2 non-Ca^{2+}-CaM-MLCK Pathways

The contraction of smooth muscle depends on extracellular Ca^{2+} and disappears following Ca^{2+} removal (Longbottom et al., 2000). Thus, the main force production is Ca^{2+}-CaM-MLCK pathway (Longbottom et al., 2000). However, there is evidence that uterine force development may be influenced by non-Ca^{2+}-CaM-MLCK dependent pathways (Kupittayanant, Burdyga and Wray, 2001). Interestingly, some agonists exert their effects to activate uterine contractility not only by Ca^{2+}-dependent but also by Ca^{2+}-independent pathways. Phosphorylation of MLCP by Rho-associated kinase (ROK) results in increase in the contraction without a change in $[Ca^{2+}]_i$ when the uterus was producing force tonically rather than phasically (Kupittayanant et al., 2001). This finding suggested that under physiological conditions, the Rho-A-ROK pathway may not important for modulation of force development in the myometrium (Kupittayanant et al., 2001; Wray et al., 2003).

In the absence of external Ca^{2+}, oxytocin produced a small tonic contraction as long as oxytocin was present. However, application of wortmannin, an inhibitor of MLCK, did not affect this contraction, suggesting that it is not produced by the phosphorylation of MLC at Serine 19 by MLCK (Longbottom et al., 2000; Wray et al., 2003).

1.2.3 Nitric Oxide-Cyclic GMP Pathway

Nitric Oxide

Nitric oxide (NO) is produced by the catalytic action of NO synthases (NOS) that convert the precursor amino acid, L-arginine, to NO and L-citrulline (Wu and Morris, 1998). NOS exists in three isoforms: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). It is indicated that L-arginine is the only physiological substrate for NOS generation (Hoffmann et al., 2003; Norman, 1996; Wu and Morris, 1998). NO binds to and activates NO-guanylate cyclase, which increases synthesis of cGMP from GTP and results in activation of PKG (Hoffmann et al., 2003; Norman, 1996). These processes provide a cascade of reaction to regulate various physiological functions. The mechanism of action of NO may cause smooth muscle relaxation through: 1) the activation of guanylate cyclase; 2) the stimulation of K_{Ca} channels; and 3) ADP ribosylation (Norman, 1996). However, the most important mechanism is due to the activation of soluble guanylate cycles, which catalyses the formation of cGMP. It has been reported that the relaxant property of NO or L-arginine is mimicked by 8-bromo-cGMP, a plasma permeable form of cGMP, and inhibited by methylene blue, an inhibitor of guanylate cyclase (Izumi and Garfield, 1995; Norman, 1996). In addition, the relaxant action of NO may be due to the activation of K_{Ca} channels. Inhibition of K_{Ca} channels results in increase of force development, an increase in $[Ca^{2+}]_i$ and a reduction in the cell membrane potential. Analogues of L-arginine, such as N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine methyl ester (L-NAME), inhibit the effect of NOS by competitive inhibition *in vivo* (Norman, 1996).

Cyclic GMP

As mentioned above, cGMP is the second messenger of NO produced by soluble guanylate cyclase. The action of cGMP is thought to be due to the inhibition of pharmacological coupling and inhibition of the contractile system. In addition, it is indicated that the mechanism for cGMP-dependent relaxation pathway is involved in the activation of a Ca^{2+} extrusion system. The pharmacological agents that generate cGMP and PKG can be used as the relaxant mediators in smooth muscle (Somlyo and Somlyo, 1998). There is evidence that mechanisms for cGMP-mediated decrease in $[Ca^{2+}]_i$, including: 1) hyperpolarization by opening K^+ channels (White, Kryman, El-Mowafy, Han and Carrier, 2000); 2) reduced Ca^{2+} influx with a constant membrane potential (Anwer et al., 1992; White et al., 2000); and 3) increased in Ca^{2+} efflux and/or Ca^{2+} sequestration (Rembold, 1995; White et al., 2000). Recently, it has been demonstrated that cGMP-induced relaxation is predominantly on the inhibition of $G_{q/11}$/PLCβ/IP_3 pathway (Izumi and Garfield, 1995).

Potassium Channels

It is well known that resting membrane potential is modulated by the K^+ conductance of the membrane (Brainard et al., 2007; Wray et al., 2003). It is indicated that the biological functions of K^+ channels are maintenance of the resting membrane potential, termination of the action potential, modulation of the electrolyte balance (Brainard et al., 2007). Many different types of K^+ channels have been identified in the cell membrane of smooth muscle cells (Parkington and Coleman, 1990). The K_{Ca} channels are one of the different categories of K^+ channels found in the myometrium

(Anwer et al., 1992; Buxton, 2004). They are activated by an increase in $[Ca^{2+}]_i$. The K_{Ca} channels play a key role in modulating the action potential and controlling the cell membrane excitability (Anwer et al., 1992; Norman, 1996). It has been reported that NO itself and PKG appear to be activated K_{Ca} channels to promote relaxation (Brainard et al., 2007; Izumi and Garfield, 1995). Periods of uterine quiescence throughout pregnancy are predominantly associated with an increase in K_{Ca} channel expression. Conversely, during labor, the expression of K_{Ca} channels appears to be decreased (Anwer et al., 1992; Wray et al., 2003).

1.3 Watermelon

Medicinal plants have been used with varying success to treat and prevent diseases throughout history. In developing countries, ethnobotanical remedies serve as their primary medicines, leaving almost 75% of the world population without access to the modern healthcare products. In developed counties, many important industrial and pharmaceutical products are either extracted from medicinal plants directly or synthesized from precursors that are extracted from plants (Souza Brito, 1996).

Watermelon (*Citrullus lanatus*.), family Cucurbitaceae, refers to both fruit and plant of a trailing herb originally from southern Africa and one of the most common types of watermelon. Phytochemical studies indicate that watermelon contains high levels of citrulline, lycopene, and β-carotene (Perkins-Veazie, Collins, Clevidence and Wu, 2007). It has been used for treatment of various ailments and improvement of sexual function in man. Rao, Ray and Rao (2006) indicated that lycopene in watermelon, an antioxidant carotenoid, can prevent the prostate cancer, osteoporosis,

hypertension, and also male infertility. As mentioned above, watermelon is an excellent source of citrulline, an amino acid of great interest because citrulline can be transformed to arginine via argininosuccinate synthase and lyase in all animal cells (Maarsingh, Leusink, Zaangsma and Meurs, 2006). Arginine is required for the formation of NO which is released during sexual stimulation by parasympathetic neurons in the penis and also by the endothelial cells lining the blood vessels and the lacunar spaces of the corpus cavernosum (Toda, Ayajiki and Okamura, 2005). NO activates soluble guanylate cyclase leading to an increased conversion of GTP to cGMP, which provides the signal for smooth muscle relaxation, including corpus cavernosum of the penis (Toda et al., 2005) and the myometrium (Izumi and Garfield, 1995; Norman, 1996).

Furthermore, watermelon is one of few foods rich in lycopene, a non provitamin A carotenoid that has up to twice the antioxidant capacity of β-carotene *in vitro* (Miller, Sampson, Candeias, Bramley and Rice-Evans, 1996). Data from epidemiological studies suggest lycopene may have protective effects against certain types of cancers and cardiovascular diseases (Rimando and Perkins-Veazie, 2005). Wu et al. (2007) used Zucker diabetic fatty (ZDF) rats as an animal model of noninsulin-dependent diabetes mellitus. ZDF rats received L-arginine 0.24%, watermelon juice 63%, lycopene 0.01%, or citrus pectin 0.05% and water served as a negative control for 4 weeks supplementation period. Heart, aortic ring and blood sample were collected for physiological analysis. The results exhibited that the groups which received watermelon juice and L-arginine reduced fat accretion; increased serum concentrations of arginine; lowered serum concentrations of glucose, free fatty acids, homocysteine, and dimethylarginines; enhanced GTP cyclohydrolase-I activity

and tetrahydrobiopterin concentrations in the heart; and improved acetylcholine-induced vascular relaxation, when compared with the control group. These results provide the knowledge for beneficial effect of watermelon juice as a functional food for increasing reducing serum concentrations of cardiovascular risk factors arginine availability, improving glycemic control and ameliorating vascular dysfunction in obese animals with type-II diabetes.

In addition, Raghavan and Dikshit (2001) investigated the relaxant effects of L-citrulline in phenylephrine pre-contracted endothelium intact thoracic aortic rings compared between control and lipopolysaccharide-treated rats. The result indicated that L-citrulline significantly increased the relaxant effects on both control and lipopolysaccharide-treated rings by supplementing the release of NO due to its recycling to L-arginine. Interestingly, arginine in watermelon is used in the NO pathway to help in vasodilatation and overall cardiovascular health (Collins et al., 2007). Anti-allergic potential activity of watermelon has also been postulated (Tabata, Cho, Shimakura and Ito, 1993).

1.4 Aims

To the best of our knowledge, the tocolytic effects of watermelon on rat uterine contraction have not yet been elucidated. As there is a clinical need to find better drugs with fewer undesirable side effects to modulate uterine activity. One of such plants claimed to have tocolytic potential is watermelon (*C. lanatus*). Thus, this thesis aimed; 1) to investigate the effects of the extracts from watermelon on rat uterine contractions; 2) to investigate the mechanisms whereby the extracts exerted

their effects; and 3) to examine whether the effects of the extracts were due to their major constituents, L-citrulline and/or L-arginine.

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CHAPTER II

GENERAL MATERIALS AND METHODS

This chapter will provide a general description of the equipment, materials and methodology performed in the work represented in this thesis. More details important to each study are presented in the individual chapter concerned.

2.1 Plant Preparation

2.1.1 Plant Material Collection

The fruits of watermelon (Figure 2.1) were collected in the Province of Nakhon Ratchasima, Thailand, where the plant was cultivated under natural conditions. Voucher specimen (BKF No. 165073) was identified and deposited at the Royal Forest Department of Thailand, Bangkok, Thailand.

2.1.2 Plant Extraction

Watermelons were cleaned and cut transversely between blossoms and stem ends. Fruit flesh, rind, and peel were separated. Flesh samples were collected from center, locule, and heart. Rind samples were collected from the white area of the fruit.

Peel samples (approximately 2 mm thickness) were removed by peeler before being chopped into pieces approximately 1 × 3 × 7 mm^3. Dried watermelon flesh (500 mg) and rind (500 mg) were subjected to Soxhlet extraction with 70% ethanol (500 mL) for 24 h. The extracts were filtered using Whatman paper No. 1. The filtrates were evaporated in a rotary evaporator under reduced pressure and low temperature. The resultant residue was lyophilized by lyophilizer and kept at -20°C in a refrigerator until use (Figure 2.2). The yields were 45% for flesh and 65% for rind (w/w based on the dried starting weight), respectively.

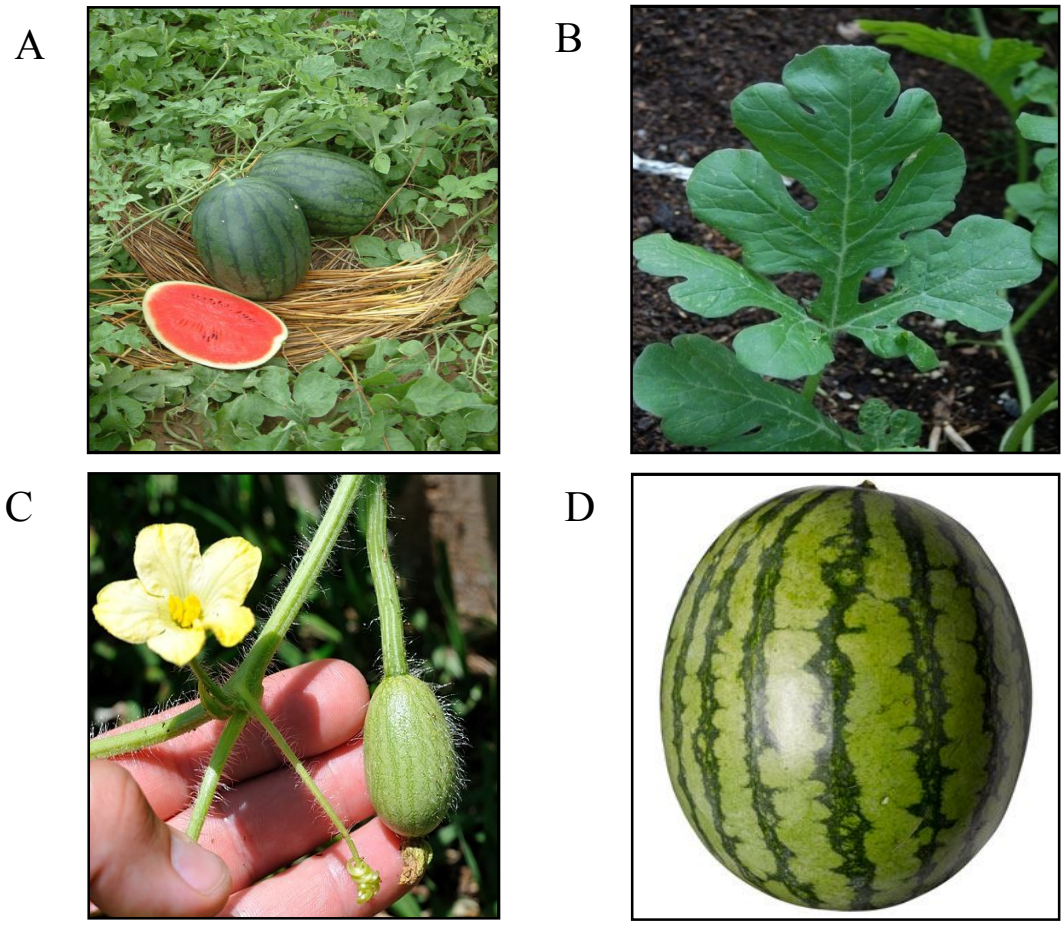

Figure 2.1 Morphology of watermelon (*C. lanatus*) (A), leaves (B), flower and pulp (C), and fruit (D).

A

B

C

Figure 2.2 The apparatus used in the extraction process. (A) Soxhlet extractor, (B) rotary evaporator and (C) lyophilizer.

2.2 Animal Preparations

2.2.1 Animal Ethics and Regulations

In this thesis, animal care and use were conducted in accordance with guidelines of the Committee on Care and Use of Laboratory Animal Resources, National Research Council of Thailand. The procedures of the experiment were performed in accordance with the advice of the Institutional Animal Care and Use Committee, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

2.2.2 Housing

Female Wistar rats were used for the present experiments. Rats were individually housed in 24 × 15 × 15 cm^3 cages under a 12:12-hour light-dark illumination cycles at a constant temperature of 25 ± 0.5°C and humidity (45-50%). Rats were given a standard laboratory food containing 0.8% calcium (CP. Co. Ltd., Thailand), and provided to water *ad libitum*.

2.2.3 Myometrial Tissue Preparations

Female Wistar rats (200-250 g) were sacrificed by asphyxiation with CO_2 and the uterine horns were isolated. Uterine strips were dissected from both cervical and ovarian segments of each uterine horn, immediately placed in Krebs' solution of the following composition (mM): NaCl: 154.0; KCl: 5.4; $CaCl_2$: 2.0; $MgSO_4$: 1.2; glucose: 8.0; HEPES: 10.0 (37°C, pH = 7.4).

2.2.4 Measurements of Tension

The uterus strip was cut into longitudinal strips of 1-2 mm × 0.5 mm × 10 mm and attached at each end to metal hooks and another hook was fixed to a transducer (AD Instruments Pty Ltd., Spain) in the organ bath that contained Krebs' solution (37°C, pH = 7.40). An equilibration time of 30 min was applied for all tissues before the application of any chemical studied. The change in isometric force was measured during 10 min with PowerLab system software (AD Instruments Pty Ltd., Australia). The relaxation was expressed as a percentage of reduction of the contractile tension induced by each agonist. The effects were measured as changes in isometric force and recorded with a force-displacement transducer connected to a computer using Chart software (Figure 2.3).

Figure 2.3 Representation of the equipment used for tension measurement. (A) Monitor, (B) CPU, (C) Keyboard, (D) Thermostat, (E) Force Transducer, (F) Organ Bath, (G) Bridge Amplifier, (H) PowerLab System and (I) Peristaltic Pump.

2.3 Chemicals

Most chemicals were obtained from Sigma®, Singapore. All the stock solutions were prepared and stored in accordance with the guideline of the producer. A KCl (40 mM) Krebs' solution was made by isoosmotic replacement of sodium chloride (Noble and Wray, 2002). Oxytocin was dissolved in distilled water and used at concentration of 10 nM to produce a phasic contraction (Kupittayanant, Luckas, and Wray, 2002). Prostaglandin $F_{2\alpha}$, ($PGF_{2\alpha}$-tris; (5Z,9_{α},11_{α},13E,15)-9,11,15-trihydroxyprosta-5,13-dienoic acid tris salt), was dissolved in the absolute ethanol and used at the concentration of 1 μM (Buddhakala, Talubmook, Sriyotha, Wray and Kupittayanant, 2008). Watermelon flesh (6 mg/mL) or rind (5 mg/mL) extract was used and dissolved in physiological solution. The positive control agents, L-citrulline (64 μM) and L-arginine (104 μM), were dissolved in physiological solution directly. N^{G}-nitro-L-arginine methyl ester (L-NAME), a non-selective inhibitor of NOS, was dissolved in distilled water and used at a concentration of 100 μM (Yallampalli, Garfield and Byam-Smith, 1993). LY 83583, the inhibitor of guanylate cyclase, was dissolved in distilled water and used at a concentration of 1 μM (Bradley, Buxton, Barber, McGaw and Bradley, 1998). Tetaethylamonim chloride, an inhibitor of calcium-dependent potassium channels (TEA) was dissolved in distilled water and used at a concentration of 5 mM (Kupittayanant et al., 2002). Bay K8644, the L-type Ca^{2+} channel activator; S-(−)-1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)-phenyl]-3 pyridinecarboxylic acid methyl ester, was dissolved in absolute ethanol and used at the concentration of 1 μM (Kupittayanant, Kupittayanant and Suwannachat, 2008). In some experiments 0-Ca solutions were used; physiological solution in which

$CaCl_2$ had been omitted and ethylene glycol tetraacetic acid (EGTA, 1 mM) added (Kupittayanant et al., 2002). The physiological Krebs' solution (pH = 7.4) contained the following (mM): NaCl 154.0, KCl 5.4, $MgSO_4$ 1.2, glucose 8.0, $CaCl_2$ 2.0, and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 10.0.

2.4 Statistical Analysis

The data were analyzed using Microcal Origin Software, Singapore. The following parameters of contraction were measured; force integral, frequency, and amplitude. The phasic contractions in the extracts were measured over 30 min after their application. Results were expressed as percentages of control contractions (i.e. the control is 100%). To test the effects of applications of L-NAME, LY 83583, Bay K8644, 5 mM $CaCl_2$, or TEA following the extracts, contractions were compared for the 30 min in the extracts (i.e. 30-60 min after start of the extract exposure), to the 60-90 min in the extracts with an addition of L-NAME, LY 83583, Bay K8644, 5 mM $CaCl_2$, or TEA. Integrated force (area under the contraction) was measured. In some experiments, the maximal tension of oxytocin- or $PGF_{2\alpha}$-induced contractions in the absence of the external calcium in the control group (without the extracts or positive control agents) was considered as 100%. Data were presented as mean ± S.E.M. and "n" represents the number of sample, each one from a different animal. Significance was tested using appropriate *t*-test. The $P < 0.05$ was considered to be significant. Results were then expressed as percentages of control contractions (i.e. the control is 100%). Dose response curves were plotted by using a nonlinear curves fitting program, Microcal Origin Software (Vergara-Galicia et al., 2010).

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CHAPTER III

DOSE DEPENDENCY OF WATERMELON (*CITRULLUS LANATUS*) EXTRACTS AND OBSERVATIONS ON SPONTANEOUS CONTRACTION

3.1 Abstract

Watermelon (*Citrullus lanatus*) is rich in L-citrulline and L-arginine, substrates for nitric oxide synthase in the production of a potential vasodilator, nitric oxide (NO). However, the effects of watermelon on uterine smooth muscle have not been studied. Thus, this study was aimed to investigate dose dependency of watermelon extracts on uterine contraction. Watermelon flesh and rind were ethanolic extracted. Isometric force was measured in isolated rat uterine strips and cumulative concentrations of watermelon extracts on spontaneous contractions examined. The results showed that watermelon flesh and rind (2-8 mg/mL) inhibited spontaneous contraction. The EC_{50} values of flesh and rind were 6 mg/mL and 5 mg/mL, respectively. The flesh extract is less potent than that the rind extract, and this might be due to lower amount of L-citrulline and L-arginine.

3.2 Introduction

The World Health Organization survey indicates that 70-80% of world populations have utilized a non-conventional medicine mainly of herbal sources in their therapeutic proposes (Chan, 2003). Plants have importantly served as a source of investigation for novel drug compounds and plant medicines have supported large contributions to human health and well being. The use of herbal plants is very common in developing countries, particularly in rural settings. During the last decade, an increase in the use of plants has been revealed in metropolitan areas of developed countries (Harnack, Rydell and Stang, 2001). The prevalence of harmful side effects from plant derived medicines, when used in an appropriate procedure, are relatively less frequent when compared to synthetic drugs. However, clinical data are still required to assess their safety (Calixto, 2000).

Some plants and their phytochemical constituents have been reported to have a beneficial effect on modulation of uterine contractility (Buddhakala, Talubmook, Sriyotha, Wray and Kupittayanant, 2008; Lijuan, Kupittayanant, Chudapongse, Wray and Kupittayanant, 2011; Promprom, Kupittayanant, Indrapichate, Wray and Kupittayanant, 2010). It is well known that medicinal plants are present as numerous chemical constituents, which usually exert their therapeutic effects through muti-targets and muti-pathways (Cao et al., 2008). The nature of these actions results in either the stimulation (uterotonic) or inhibition (tocolytic) of uterine smooth muscle contractions. As there is a clinical need to find better drugs with fewer undesirable side effects to inhibit uterine activity (Buddhakala et al., 2008), one of such plants claimed to have tocolytic potential such as watermelon (*C. lanatus*) (Figueroa,

Sanchez-Gonzalez, Perkins-Veazie and Arjmandi, 2010; Jayaprakasha, Murthy and Patil, 2011) has been receiving attention.

Watermelon (*C. lanatus*) belongs to the family of Cucurbitaceae and is a native tropical and subtropical plant and extensively cultivated in Africa, the United States, India, China, and Thailand (Robinson and Decker-Walters, 1997). The plant is characterized by a spreading, hairy, tendril-bearing vine, 3-5 meters in length. The stems are thin, hairy, angular, grooved, and have branched tendrils at each node. Leaves are oblong-ovate 8-20 cm long containing 3-7 lobes. Flowers of watermelon are 2 cm in diameter, monoecious, yellow in color. Fruits are large, green-mottled or deep green, round to cylindrical and have a rind 10 to 40 mm thick. Roots are extensive but shallow, with a tap root and many lateral roots (Robinson and Decker-Walters, 1997). Watermelon is commonly known in Thai as Taeng-Mo. Its fruit has been consumed as both dessert fruit and vegetable proposes. Some chemical constituents and biological activity of watermelon have been reported (Figueroa et al., 2010; Jayaprakasha et al., 2011). Watermelon is one of the few foods naturally rich in specific amino acids, L-citrulline and L-arginine (Tlili, Hdider, Lenucci, Ilahy, Jebari and Dalessandro, 2011a; Wu et al., 2007). Dietary antioxidants such as carotenoids and phenolic compounds have been isolated. These secondary metabolites have been found to be effective in promoting peroxyl-radical scavenging activity, reducing the risk of some types of cancers, cardiovascular diseases and age-related degenerative pathologies (Tlili, Hdider, Lenucci, Ilahy, Jebari and Dalessandro, 2011b; Wu et al., 2007). It was found that watermelon juice can protect against CCl_4-induced hepatotoxicity and oxidative stress in rats (Altas, Kızıl, Kızıl, Ketani and Haris,

2011). In addition, watermelon supplement has a beneficial effect on prehypertension (Figueroa et al., 2011) and diabetes (Ahn, Choi, Kim and Ha, 2011; Wu et al., 2007).

As the ethnopharmacological potential described to watermelon is largely based on empirical data, more research is required to scientifically prove its action and efficacy. It has been reported that watermelon extract induces the relaxation of smooth muscle cells through NO pathway modulation and reduction of intracellular Ca^{2+} $[Ca^{2+}]_i$ (Jayaprakasha et al., 2011). However, no measurements of its effects on agonists-induced contractions have been made so far in isolated rat uteri. In addition, the mechanisms underlying contractility changes are incompletely understood. Thus, this study was aimed to investigate dose dependency of watermelon flesh and rind extracts on spontaneous uterine contractions using isolated rat uteri.

3.3 Materials and Methods

3.3.1 Myometrial Tissue Preparations and Measurements of Tension

Myometrial tissue preparations were dissected and force measurements measured as those described in Chapter II (Sections 2.2.3 and 2.2.4, respectively).

3.3.2 Dose Dependency of Watermelon Extracts

Following a 30-min equilibration time, watermelon flesh or rind extract (2-8 mg/mL) was added into the bathing solution in a cumulative increase in concentration manner. The median effective concentrations (EC_{50} values, concentration required to produce 50% of the maximum inhibition of the amplitude of

contraction) were calculated by using a nonlinear curves fitting program, Microcal Origin Software (Vergara-Galicia et al., 2010).

3.3.3 Chemicals and Physiological Solution

All chemicals were purchased from Sigma®, Singapore. The measurement of tension was made whilst the tissue was continually perfused with physiological solution (control).

3.3.4 Preparation of Watermelon Flesh and Rind Extracts

As described in Chapter II (Section 2.1.2), stocks of watermelon extracts were kept at -20°C. Fruit flesh or rind extract was dissolved in Krebs' solution just before use.

3.3.5 Statistical Analysis

The data were analyzed using Microcal Origin Software. The integral force was used as the parameter of contraction. Results were expressed as percentages of control contractions (i.e. the control is 100%). Throughout, data are presented as mean ± S.E.M. and "n" represents the number of samples, each one from a different animal. Significances were tested using appropriate *t* tests. The P value < 0.05 was taken to be significant.

3.4 Results

3.4.1 Dose Dependency of Watermelon Flesh Extract

To investigate whether the effect of flesh extract was dose-dependent, 2, 4, 6, or 8 mg/mL of flesh extract was added to the phasic spontaneous contractions in a cumulative increase in concentration manner. It can be seen that greater decrements of force occurred as the dose of watermelon flesh extract increased. Compared with control peak amplitude (100%), the mean amplitudes were reduced to 91.49 ± 7.55%, 82.61 ± 9.37%, 55.64 ± 9.05%, and 8.48 ± 5.64% with 2, 4, 6, and 8 mg/mL flesh extract, respectively. The cumulative concentrations of watermelon flesh extract also induced an increased frequency of uterine contractions. As shown in Figure 3.1, the EC_{50} value of flesh extract was 6.12 ± 1.02 mg/mL. Therefore, the concentration of 6 mg/mL was selected and used throughout the remainder of the study. The inhibitory effects of cumulative doses of watermelon flesh extract on the amplitude, frequency, and AUC of contractions are summarized in Table 3.1.

3.4.2 Dose Dependency of Watermelon Rind Extract

After the equilibration period, the uterine strip was exposed to cumulative doses of rind extract (2, 4, 6, or 8 mg/mL). It was found that the addition of increasing concentrations of rind extract produced a marked decrease in the amplitude of contractions compared with control (100%). The amplitudes produced by rind extract measured after 10 min of application were 92.98 ± 7.94%, 72.27 ± 9.35%, 41.81 ± 7.65%, and 6.57 ± 3.14% with 2, 4, 6, and 8 mg/mL, respectively. The cumulative concentrations of watermelon rind extract induced an increased frequency of uterine

contractions. As shown in Figure 3.1, the EC_{50} value of rind extract was 5.26 ± 1.08 mg/mL. Therefore, the concentration of 5 mg/mL was selected and used throughout the remainder of the study. The inhibitory effects of cumulative doses of watermelon rind extract on the amplitude, frequency, and AUC of contractions are summarized in Table 3.2.

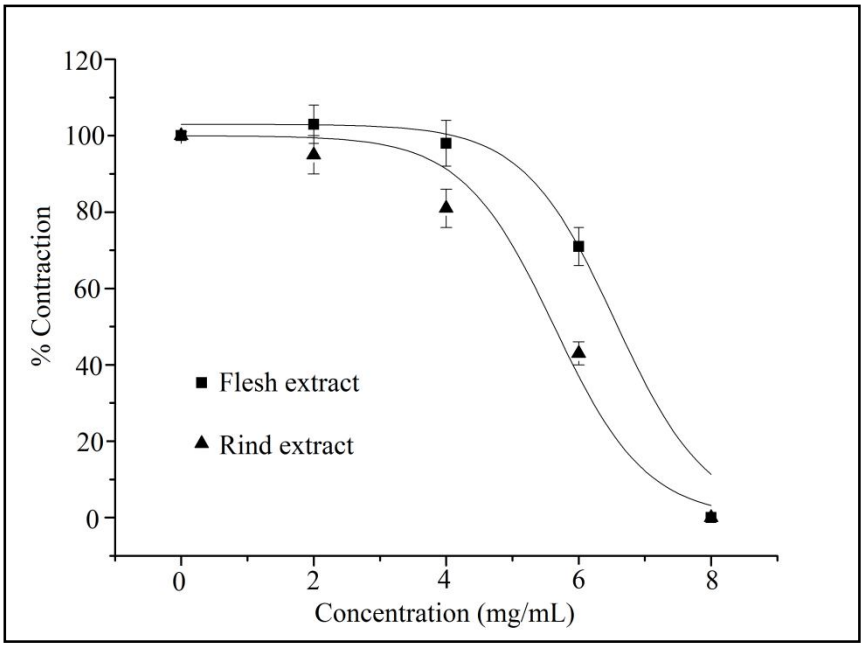

Figure 3.1 Dose dependency of watermelon flesh and rind extracts. The effects of increasing cumulative concentrations of flesh and rind extracts (2-8 mg/mL) on spontaneous contractions of non-pregnant rat uterus are shown. Symbol represents means. Vertical lines represent standard errors of the means (n = 6 for each extract).

Table 3.1 The effects of watermelon flesh extract at various concentrations on spontaneous contraction.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
Watermelon flesh (mg/mL)				
0 (Control)	100	100	100	6
2	91.49 ± 7.55	125.57 ± 4.90*	95.38 ± 5.90	6
4	82.61 ± 9.37	120.18 ± 1.94*	85.10 ± 6.22	6
6	55.64 ± 9.05*	115.55 ± 3.21*	63.12 ± 4.49*	6
8	8.48 ± 5.64*	8.61 ± 5.26*	3.94 ± 2.55*	6

The *P*-values for amplitude, frequency and AUC of watermelon flesh extract performed are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

Table 3.2 The effects of watermelon rind extract at various concentrations on spontaneous contraction.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
Watermelon rind (mg/mL)				
0 (Control)	100	100	100	6
2	92.98 ± 7.94	124.56 ± 1.03*	92.98 ± 7.94	6
4	72.27 ± 9.35*	119.64 ± 8.65*	72.27 ± 9.35*	6
6	41.81 ± 7.65*	113.35 ± 1.35*	41.81 ± 7.65*	6
8	6.57 ± 3.14*	10.76 ± 1.32*	6.59 ± 3.14*	6

The *P*-values for amplitude, frequency and AUC of watermelon rind extract performed are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

3.4.3 Effects of Watermelon Flesh Extract on Spontaneous Contraction

The application of watermelon flesh extract (6 mg/mL) to the rat myometrial preparations produced a significant decrease in the amplitude of force (67.52 ± 6.43% compared with control, 100%, $P < 0.05$). As shown in Figure 3.3, the frequency of the contraction produced by flesh extract was significantly increased to 116.25 ± 4.70% when compared with control (100%, $P < 0.05$). 30 min later, the uterine strip was then washed with Krebs' solution. It was found that the phasic spontaneous contractions of the uterus were reversed. Thus, this finding indicates that the tocolytic effects of flesh extract on the uterus were reversible. The effects of watermelon flesh extract at the dose of 6 mg/mL on spontaneous contraction are summarized in Table 3.3.

3.4.4 Effects of Watermelon Rind Extract on Spontaneous Contraction

The application of watermelon rind extract (5 mg/mL) to the rat myometrial preparations produced a significant decrease in the amplitude of force (58.02 ± 7.68% compared with control, 100%, $P < 0.05$). As shown in Figure 3.4, the frequency of the contraction produced by rind extract was significantly increased to 115.49 ± 4.26% when compared with control (100%, $P < 0.05$). 30 min later, the uterine strip was then washed with Krebs' solution. It was found that the phasic spontaneous contractions of the uterus were reversed. Thus, this finding indicates that the tocolytic effects of rind extract on the uterus were reversible. The effects of watermelon rind extract at the dose of 5 mg/mL on spontaneous contraction are summarized in Table 3.3.

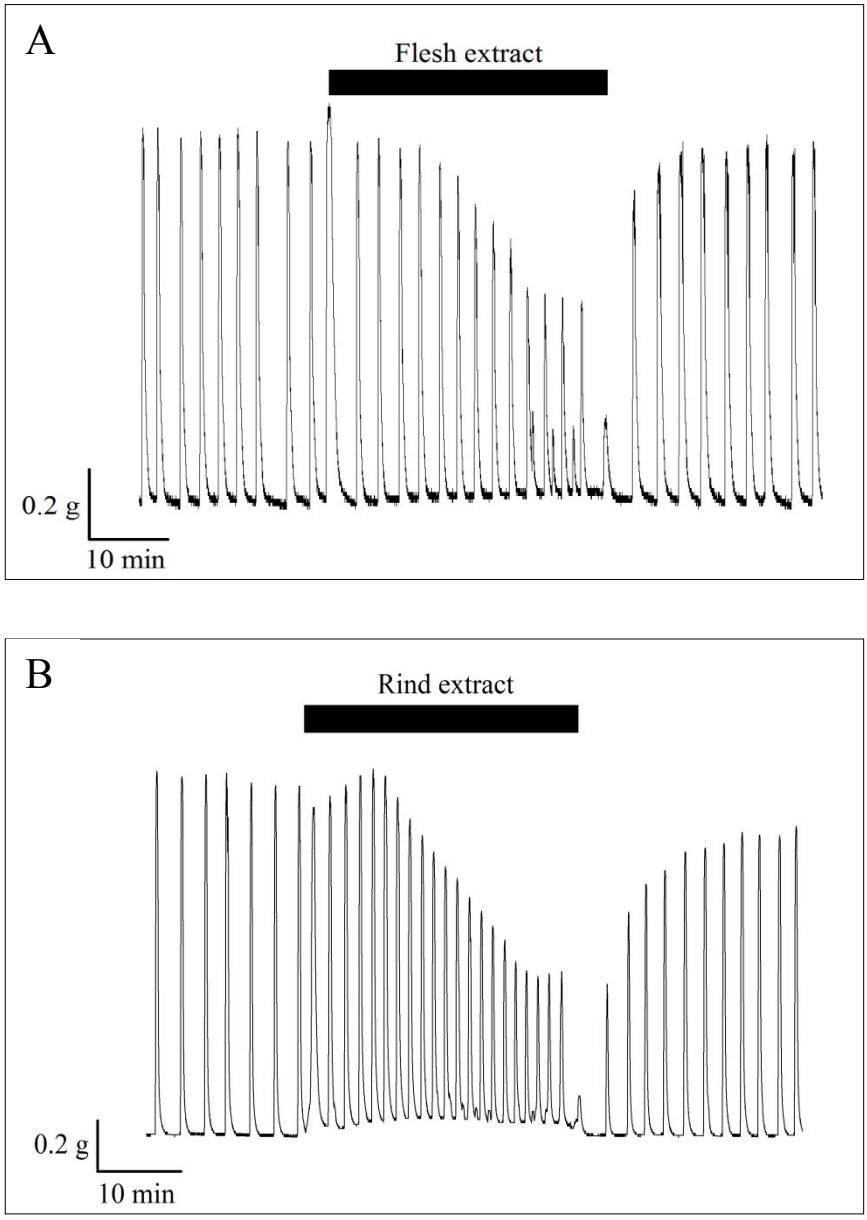

Figure 3.2 The effects of watermelon flesh (6 mg/mL; A) and rind (5 mg/mL; B) extracts on spontaneous contractions (n = 6 for each extract).

Table 3.3 The effects of watermelon flesh (6 mg/mL) and rind (5 mg/mL) extracts on spontaneous contraction.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
Watermelon flesh				
Control	100	100	100	6
Watermelon flesh	67.52 ± 6.43*	116.25 ± 4.70*	70.44 ± 4.10*	6
Recovery	94.25 ± 5.37	99.11 ± 3.12	94.42 ± 7.27	6
Watermelon rind				
Control	100	100	100	6
Watermelon rind	58.02 ± 7.68*	115.49 ± 4.26*	66.63 ± 2.83*	6
Recovery	90.63 ± 7.39	100.00 ± 4.02	96.77 ± 3.75	6

The *P*-values for amplitude, frequency and AUC of watermelon flesh and rind extracts performed are significantly different from the control ($^{*}P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

3.5 Discussion

This study is the first to investigate the dose-dependent effects of watermelon extracts on rat uterine contraction. The data show that the inhibitory effects of watermelon flesh and rind extracts (2-8 mg/mL) were dose-dependent. The EC_{50} values of flesh and rind extracts were 6.13 ± 1.35 and 5.26 ± 1.08 mg/mL, respectively. The effects of flesh extract at the dose of 6 mg/mL and rind extract at the dose of 5 mg/mL were also examined. It was found that at these concentrations, both flesh and rind extracts were significantly decreased in the amplitude and the mean integral force but not the frequency of the contractions.

It is generally accepted that Ca^{2+} ion plays a crucial role in modulation of force development (Somlyo and Somlyo, 1994). The increase of $[Ca^{2+}]_i$ for the myometrium contraction comes from two sources: the extracellular through L-type Ca^{2+} channels and the intracellular source from the SR. It has been reported that uterine smooth muscle contraction can be generated by several mechanisms, but the main mechanism depends on Ca^{2+}-calmodulin (CaM)-myosin light chain kinase (MLCK) pathway (Longbottom, Luckas, Kupittayanant, Badrick, Shmigol and Wray, 2000).

The watermelon flesh and rind (2-8 mg/mL) extracts caused an inhibition of spontaneous contractions in dose-dependent manner. In addition, the application of flesh (6 mg/mL) and rind (5 mg/mL) to spontaneously generated contractions of uterine smooth muscle produced a marked decrease in the mean integral force and the amplitude of contractions. Interestingly, the amplitude of the contractions produced by flesh was less than that produced by rind. Thus, this finding suggests that

watermelon extracts can exert their effects in rat isolated uterine strips by inhibiting Ca^{2+} influx and some of the Ca^{2+} signaling element involved in the smooth muscle contraction. It has been reported that some agents which reduce spontaneous contractile activity 1) may be associated with the decrease of Ca^{2+} entry and/or 2) may have an influence on mechanisms which follow the influx of Ca^{2+} (Wray, Duggins, Ilfs, Nyman and Osman, 1992). Thus, it is possible to speculate that the plant extracts may depress force production through the inhibition of extracellular Ca^{2+} influx which is mediated by one or combined mechanisms as described before. There is evidence that watermelon extracts can decrease the levels of $[Ca^{2+}]_i$ by using Flou-4 AM dye, a specific fluorescent probe for $[Ca^{2+}]_i$, in smooth muscle cell line (Jayaprakasha et al., 2011). The results indicated that smooth muscle relaxation is mainly through the reduction of $[Ca^{2+}]_i$ (Jayaprakasha et al., 2011).

The application of watermelon flesh and rind extracts to spontaneous contractions increased the frequency of contractions. This finding suggests that the plant extracts may 1) increase the intrinsic pacemaking mechanism in the uterus and 2) shorten the action potential (Wray, 1993). However, the mechanism underlying of this prevalence is unclear.

In this present study, it was also found that the tocolytic potency of the watermelon rind extract was greater than that produced by watermelon flesh extract. Watermelon is a rich source of the non-essential amino acid L-citrulline in nature. It has been reported that the L-citrulline content based on a dry weight of the rind was higher than that of the flesh (Rimando and Perkins-Veazie, 2005). According to the plant extraction in the present study, the amount of L-citrulline contained in the flesh extract should be less than in the rind extract. This could be the reason why the

tocolytic potency of the rind extract in this present study was higher than that produced by the flesh extract. If the flesh extract contained very small amount of L-citrulline, other different compounds from that contained in rind extract might contribute to the actions of the flesh extract (see below).

In addition, it has been reported that L-arginine is also found in watermelon and served as the precursor for the substrate in NO production (Hayashi et al., 2005). L-citrulline produced pharmacological effects that closely resembled those of L-arginine administration and NO action. It has been exhibited that the influx of Ca^{2+} through L-type Ca^{2+} channels may be due to the influence of NO-cGMP-cGMP-dependent protein kinase (PKG) signaling pathway (Carvajal et al., 2000). The PKG phosphorylation site of L-type Ca^{2+} channel is at Serine 533 which localized in α1c subunit (Jiang et al., 2000). It is possible to hypothesize that NO produced by the plant extracts may partially inhibit Ca^{2+} entry of uterine smooth muscle via NO-cGMP-PKG signaling pathway, leading to the reduction of force (Carvajal et al., 2000; Jiang et al., 2000). From this assessment, the inhibitory effects of watermelon extracts might also be attributed to L-arginine found in the extracts (Tlili et al., 2011a; 2011b).

Medicinal plants showing NO production can be served as the tocolytic drug, which may have potential for the prevention and treatment of miscarriage and/or other disorders related to NO in reproductive function, including dysmenorrhea and preterm labor. The results of this present study could be useful for further study to define the effects of watermelon on human myometrium *in vitro*.

In conclusion, this study investigated the dose dependency of watermelon extracts on isolated rat uterus. The results suggest that watermelon extracts had a

dose-dependent relaxing effect on the isolated rat uterine strips. The inhibitory effects of watermelon extracts might be mediated through NO-cGMP-dependent pathway modulation. In addition, the plant extracts may inhibit the Ca^{2+} influx via inhibition of L-type Ca^{2+} channels possibly through cGMP signaling and decrease the production of force. Watermelon must be submitted to further studies that help to identify other major bioactive compounds and/or determine optimal conditions for possible development of phytomedicine that improved this worldwide prevalent disease.

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CHAPTER IV

EFFECTS OF WATERMELON (*CITRULLUS LANATUS*) EXTRACTS ON AGONISTS-INDUCED UTERINE CONTRACTIONS

4.1 Abstract

Watermelon (*Citrullus lanatus*) has a potent vasodilatory effect. However, the effects of watermelon on uterine contraction have not yet been elucidated. Thus, this study was aimed to investigate the effects of watermelon extracts on agonists-induced uterine contractions. The contractile responses in the presence of watermelon flesh or rind extract were recorded isometrically with a force transducer. The results showed that watermelon extracts depressed the contractions induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), oxytocin, and potassium chloride solution (KCl). They also reduced $PGF_{2\alpha}$- and oxytocin-induced uterine force in the absence of external calcium and partially inhibited contraction induced by increasing external calcium. In addition, they caused a marked decrease in tonic contractions produced by oxytocin-induced contraction in the presence of KCl. These findings suggested that watermelon had a potent tocolytic and that the inhibitory effects of watermelon extracts were via the inhibition of both Ca^{2+}-dependent and Ca^{2+}-independent pathways of force regulation.

4.2 Introduction

Plants are known to produce a variety of metabolites with novel structures and interesting biological activities. The potential use of isolated plant metabolites for the treatment of several diseases has been the subjects of a number of investigations (Jayaprakasha, Murthy and Patil, 2011; Tarazona-Díaz, Viegas, Moldao-Martin and Aguayo, 2011). Based on ethnopharmacological data, highly selective and potent antagonists or even agonists are required for the development of natural-based chemical compounds.

Watermelon is becoming increasing popular due to its potential health benefits and refreshing flavor (Tlili, Hdider, Lenucci, Riadh, Jebari and Dalessandro, 2011). Phytochemical studies indicated that watermelon contains number of essential micronutrients and vitamins (Tarazona-Díaz et al., 2011; Tlili et al., 2011). L-citrulline and L-arginine are also found in a high content (Jayaprakasha et al., 2011; Rimando and Perkins-Veazie, 2005). In addition, flesh watermelon provides a good source of carotenoids and phenolic compounds (Tarazona-Díaz et al., 2011; Tlili et al., 2011). Ethnopharmacological relevance demonstrated that watermelon supplementation can improve aortic hemodynamics in patient with prehypertension, suggesting that watermelon has a potent vasodilator (Figueroa, Sanchez-Gonzalez, Perkins-Veazie and Arjmandi, 2010). Altas, Kızıl, Kızıl, Ketani and Haris (2011) found that watermelon juice can protect against CCl_4-induced hepatotoxicity and oxidative stress in rats. Wu et al. (2007) indicated that watermelon supplementation to Zucker diabetic fatty rats, an animal model of noninsulin-dependent diabetes mellitus can increase arginine availability, decrease serum concentrations of cardiovascular

risk factors. Recently, smooth muscle relaxant effects of watermelon extracts has been reported (Jayaprakasha et al., 2011).

As indicated in Chapter III, the effects of watermelon flesh and rind extracts were dose-dependent. In addition, the application of watermelon extracts to spontaneous phasic contractions was reversible. It was of interest to verify the mechanism underlying of action of watermelon extracts on the uterine contractions-induced by agonists and KCl. Thus, the aims of this study were to investigate the effects of watermelon extracts on the agonists (e.g. $PGF_{2\alpha}$ and oxytocin)-induced and KCl-induced uterine contractions.

4.3 Materials and Methods

4.3.1 Myometrial Tissue Preparations and Measurements of Tension

Myometrial tissue preparations were dissected and force measurements measured as those described in Chapter II (Sections 2.2.3 and 2.2.4, respectively).

4.3.2 Experimental Procedures

4.3.2.1 Effects on Bay K8644- and Increasing $CaCl_2$ Concentration-Induced Uterine Contractions

To investigate whether the tocolytic effects of watermelon extracts were dependent upon external Ca^{2+}entry through voltage-gated L-type Ca^{2+} channels, Bay K8644, the L-type Ca^{2+} agonist, or 5 mM $CaCl_2$ was used. In the experiment,

Bay K8644 or 5 mM $CaCl_2$ was applied for 30 min and then flesh or rind extract added, in the continued presence of Bay K8644 or 5 mM $CaCl_2$. In addition, the experiments were done the other way round. To do so, watermelon flesh or rind extract was applied for 30 min and then Bay K8644 or 5 mM $CaCl_2$ added, in the continued presence of the extract.

4.3.2.2 Effects on $PGF_{2\alpha}$-, Oxytocin- and KCl-Induced Uterine Contractions

The effects of watermelon extracts on $PGF_{2\alpha}$-, oxytocin- and KCl-induced uterine contractions were evaluated as follows. After equilibration period in Krebs' solution, the uterine strip was stimulated with $PGF_{2\alpha}$ (1 μM), oxytocin (10 nM) or KCl (40 mM) for 40 min and then washed. 30 min later $PGF_{2\alpha}$, oxytocin or KCl was then added into the bathing solution, and 20 min later watermelon flesh or rind extract was incubated for 20 min in the continued presence of agonists or KCl. At the end of the experiment, the bathing solution was replaced by Krebs' solution and tension monitored up to 30 min. The results of these experiments were compared with the contraction without the extracts.

4.3.2.3 Effects on $PGF_{2\alpha}$- and Oxytocin-Induced Uterine Contractions in the Absence of External Ca^{2+}

The uterine strip was incubated with 0-Ca (1 mM EGTA) solution containing watermelon flesh or rind extract for 15 min, then 1 μM $PGF_{2\alpha}$ or 10 nM oxytocin was added to stimulate the release of Ca^{2+} from the sarcoplasmic reticulum (SR). The maximal tension produced by $PGF_{2\alpha}$ or oxytocin in the control group (without watermelon extracts) was considered as 100%.

4.3.2.4 Effects on Oxytocin-Induced Uterine Contractions in the Presence of KCl

The uterine strip was incubated with KCl for 15 min. Then the solution in the bath was replaced by KCl containing 10 nM oxytocin and equilibrated for 15 min. After the maximum contractile response to KCl containing oxytocin was obtained, watermelon flesh or rind extract was then applied for 15 min. At the end of the experiment, the bathing solution was replaced by Krebs' solution and tension monitored up to 30 min.

4.3.3 Chemicals and Physiological Solutions

All chemicals were purchased from Sigma®, Singapore. Bay K8644, (the L-type Ca^{2+} channel activator; S-(−)-1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-3-pyridine-carboxylic acid methyl ester), was dissolved in absolute ethanol and used at the concentration of 1 μM (Kupittayanant, Kupittayanant and

Suwannachat, 2009). A 5 mM $CaCl_2$ solution was made by increasing the concentration of Ca^{2+} from 2 to 5 mM into Krebs' solution (Buddhakala, Talubmook, Sriyotha, Wray and Kupittayanant, 2008). The agonist oxytocin was dissolved in distilled water and used at concentration of 10 nM to produce a phasic contraction (Kupittayanant, Luckas and Wray, 2002). Prostaglandin $F_{2\alpha}$, ($PGF_{2\alpha}$-tris; (5Z,9_{α},11_{α},13E,15)-9,11,15-trihydroxyprosta-5,13-dienoic acid tris salt), was dissolved in the absolute ethanol and used at the concentration of 1 μM (Buddhakala et al., 2008). A KCl (40 mM) Krebs' solution was made by isoosmotic replacement of sodium chloride (Noble and Wray, 2002). In some experiments 0-Ca solutions were used; physiological solution in which $CaCl_2$ had been omitted and ethylene glycol tetraacetic acid (EGTA, 1 mM) added (Kupittayanant et al., 2002). The physiological Krebs' solution (pH 7.4) contained the following (mM): NaCl 154.0, KCl 5.4, $MgSO_4$ 1.2, glucose 8.0, $CaCl_2$ 2.0, and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 10.0.

4.3.4 Preparation of Watermelon Flesh and Rind Extracts

As described in Chapter II (Section 2.1.2), stocks of watermelon extracts were kept at -20°C. Fruit flesh or rind extract was dissolved in Krebs' solution just before use.

4.3.5 Statistical Analysis

The data were analyzed using Microcal Origin Software. The integral force was used as the parameter of contraction. Results were expressed as percentages of control contractions (i.e. the control is 100%). Throughout, data are presented as

mean ± S.E.M. and "n" represents the number of samples, each one from a different animal. Significances were tested using appropriate *t* tests. The *P* value < 0.05 was taken to be significant.

4.4 Results

4.4.1 Effects of Watermelon Flesh and Rind Extracts on Rat Uterine Contractions in the Presence of L-type Ca^{2+} Channel Activator

Watermelon Flesh Extract

In the myometrium, L-type Ca^{2+} channels are essential for the electromechanicl coupling that initiates the extracellular Ca^{2+} cycle (Chien, Saunders and Phillippe, 1996; Wray et al., 2003). To investigate whether the tocolytic effects of watermelon flesh extract may be due to the inhibition of L-type Ca^{2+} channels, Bay K8644 (1 μM), the L-type Ca^{2+} activator, was used (Kupittayanant et al., 2009). As shown in Figure 4.1A, pretreatment of the uterine strip with Bay K8644 produced a significant increase in the contraction amplitude (135.04 ± 1.23%, $P < 0.05$) compared with spontaneous contraction (100%, n = 6). Addition of watermelon flesh extract to the myometrial strips in the continued presence of Bay K8644 produced a marked decrease in contraction amplitude and the mean integral force to 57.05 ± 5.37% and 64.17 ± 2.27%, ($P < 0.05$) respectively, compared with spontaneous contraction (100%). When Bay K8644 was added after flesh extract, it reversed the inhibitory effects of flesh extract, but the amplitude of contractions did not return to the control level. The amplitude and the mean integral force produced by the combination of flesh extract and Bay K8644 measured after 10 min application

were 81.75 ± 2.37% and 83.80 ± 2.15%, ($P < 0.05$) respectively, compared with spontaneous contraction (100%, n = 6). The samples of experimental traces are shown in Figure 4.1 and data summarized in Table 4.1.

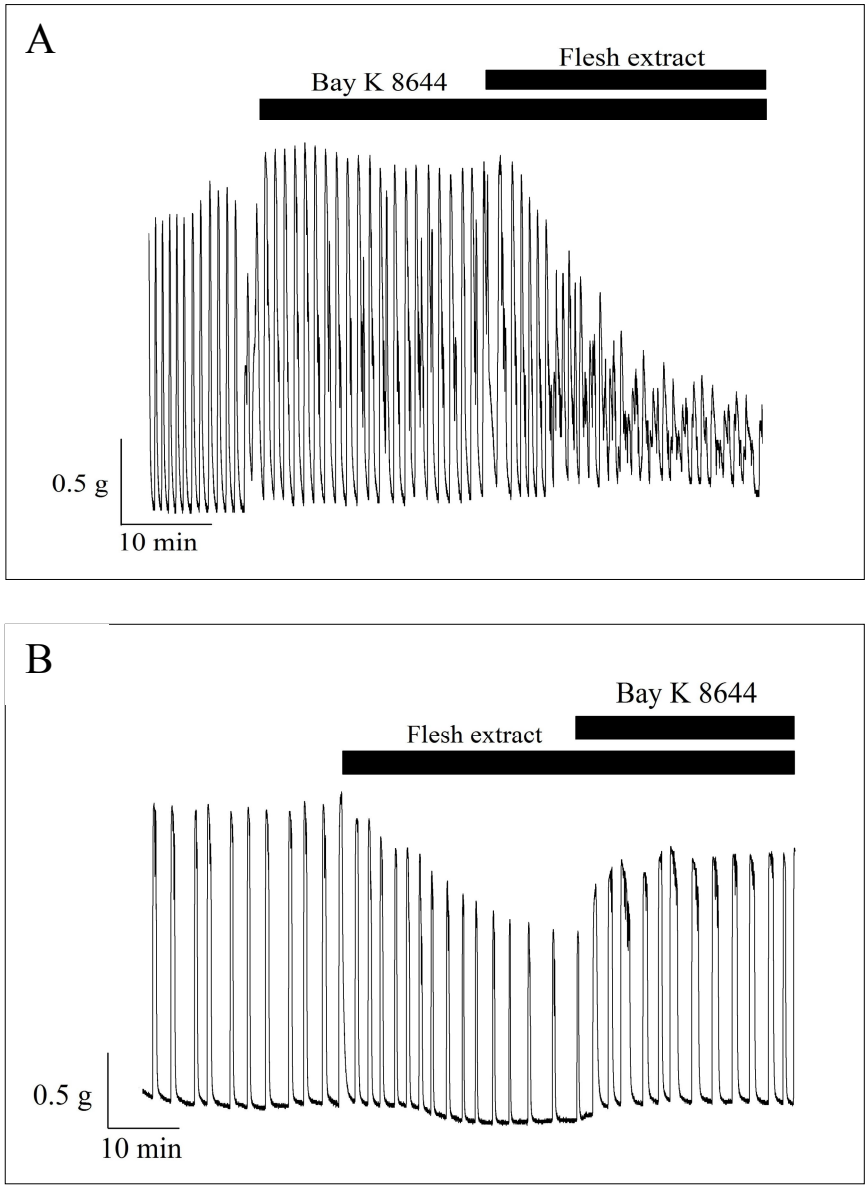

Figure 4.1 The effects of watermelon flesh extract on uterine contraction in the presence of the L-type Ca^{2+} channel activator. Bay K8644 (1 μM) was added before (A) and after (B) watermelon flesh extract (6 mg/mL; n = 6 for each).

Table 4.1 The effects of watermelon flesh extract on uterine contraction in the presence of L-type Ca^{2+} channel activator.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
Watermelon flesh (after)				
Control	100	100	100	6
Bay K8644	135.04 ± 1.23*	122.23 ± 6.70*	148.54 ± 6.70*	6
Bay K8644 + watermelon flesh	57.05 ± 5.37*	88.08 ± 6.16	64.17 ± 2.27*	6
Watermelon flesh (before)				
Control	100	100	100	6
Watermelon flesh	61.80 ± 7.73*	114.40 ± 5.00*	52.05 ± 3.87*	6
Watermelon flesh + Bay K8644	81.75 ± 2.37*	100.52 ± 5.02	83.80 ± 2.15*	6

The *P*-values for amplitude, frequency and AUC of Bay K8644 treated are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

Watermelon Rind Extract

The effects of rind extract on rat uterine contraction in the presence of Bay K8644 was also examined. As can be seen in Figure 4.2A, the application of rind extract in the continued presence of Bay K8644 caused a marked decrease of force (48.98 ± 5.57%, $P < 0.05$, compared with spontaneous contraction (100%, n = 6). The amplitude and the mean integral force produced by the combination of rind extract and Bay K8644 measured after 10 min application were 48.90 ± 5.43% and 70.35 ± 7.90%, ($P < 0.05$) respectively when compared with Bay K8644 alone. The application of Bay K8644 in the continued presence of the rind extract produced a significant increase in force. The amplitude and the mean integral force produced by the combination of rind extract and Bay K8644 measured after 10 min of application were 81.75 ± 2.37% and 80.61 ± 3.29%, ($P < 0.05$) respectively, compared with spontaneous contraction (100%, n = 6). The samples of experimental traces are shown in Figure 4.2 and data summarized in Table 4.2.

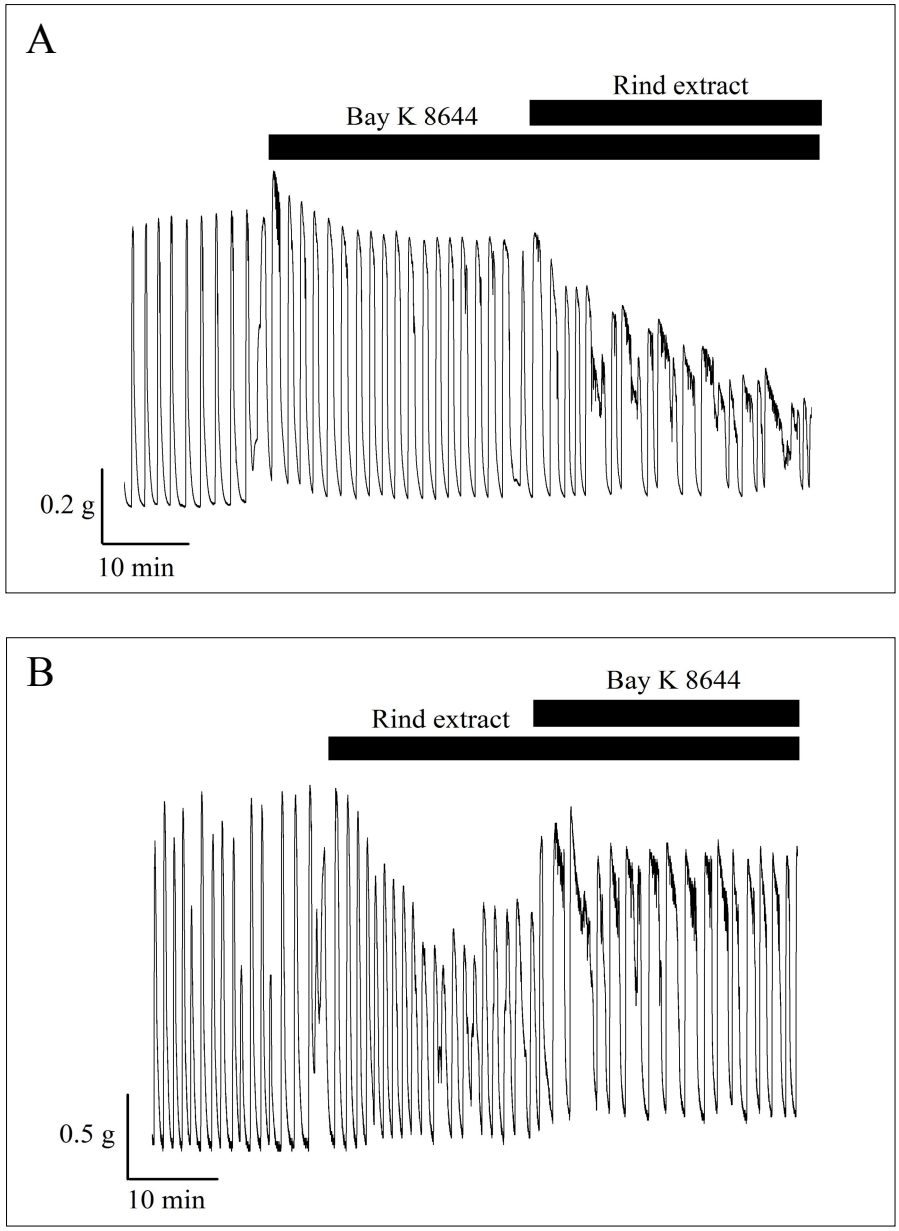

Figure 4.2 The effects of watermelon rind extract on uterine contraction in the presence of the L-type Ca^{2+} channel activator. Bay K8644 (1 μM) was added before (A) and after (B) watermelon rind extract (5 mg/mL; n = 6 for each).

Table 4.2 The effects of watermelon rind extract on uterine contraction in the presence of L-type Ca^{2+} channel activator.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
Watermelon rind (after)				
Control	100	100	100	5
Bay K8644	138.04 ± 6.43*	120.15 ± 4.75*	140.68 ± 4.49*	5
Bay K8644 + watermelon rind	48.98 ± 5.57*	84.08 ± 3.14*	64.17 ± 2.27*	5
Watermelon rind (before)				
Control	100	100	100	5
Watermelon rind	59.51 ± 4.53*	116.88 ± 3.65*	65.51 ± 3.87*	5
Watermelon rind + Bay K8644	81.75 ± 2.37*	66.63 ± 7.97*	80.61 ± 3.29*	5

The *P*-values for amplitude, frequency and AUC of Bay K8644 treated are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

4.4.2 Effects of Watermelon Flesh and Rind Extracts on Rat Uterine Contractions in the Presence of High Ca^{2+}

Watermelon Flesh Extract

It has been indicated that Ca^{2+} are important for regulating the contraction-relaxation process in smooth muscle, including the myometrium (Kupittayanant et al., 2002; Wray et al., 2003). Thus, it was of interest to investigate whether the inhibitory effects of watermelon is dependent upon external Ca^{2+} entry. To do so, the solution was changed to one with 5 mM $CaCl_2$ (Buddhakala et al., 2008) and then watermelon flesh extract added. As can be seen in Figure 4.3A, the application of high Ca^{2+} solution substituted for normal Krebs' solution (2 mM $CaCl_2$) caused a significant increase in force. The addition of watermelon flesh extract (6 mg/mL) to the uterus in the presence of high Ca^{2+} solution produced a marked rapid fall of force. The amplitude and the mean integral force of the contractions were 68.76 ± 3.40% and 67.29 ± 2.93%, ($P < 0.05$) respectively, compared with spontaneously contracting uterus (100%, n = 5). As expected, the application of high Ca^{2+} solution in the presence of flesh extract partially revered the inhibitory effects (Figure 4.3B). The amplitude of force produced by the combination of flesh extract and high Ca^{2+} solution measured after 10 minutes of application was 86.73 ± 2.71% ($P < 0.05$), when compared with spontaneous contraction (100%, n = 5). The samples of experimental traces are shown in Figure 4.3 and data summarized in Table 4.3.

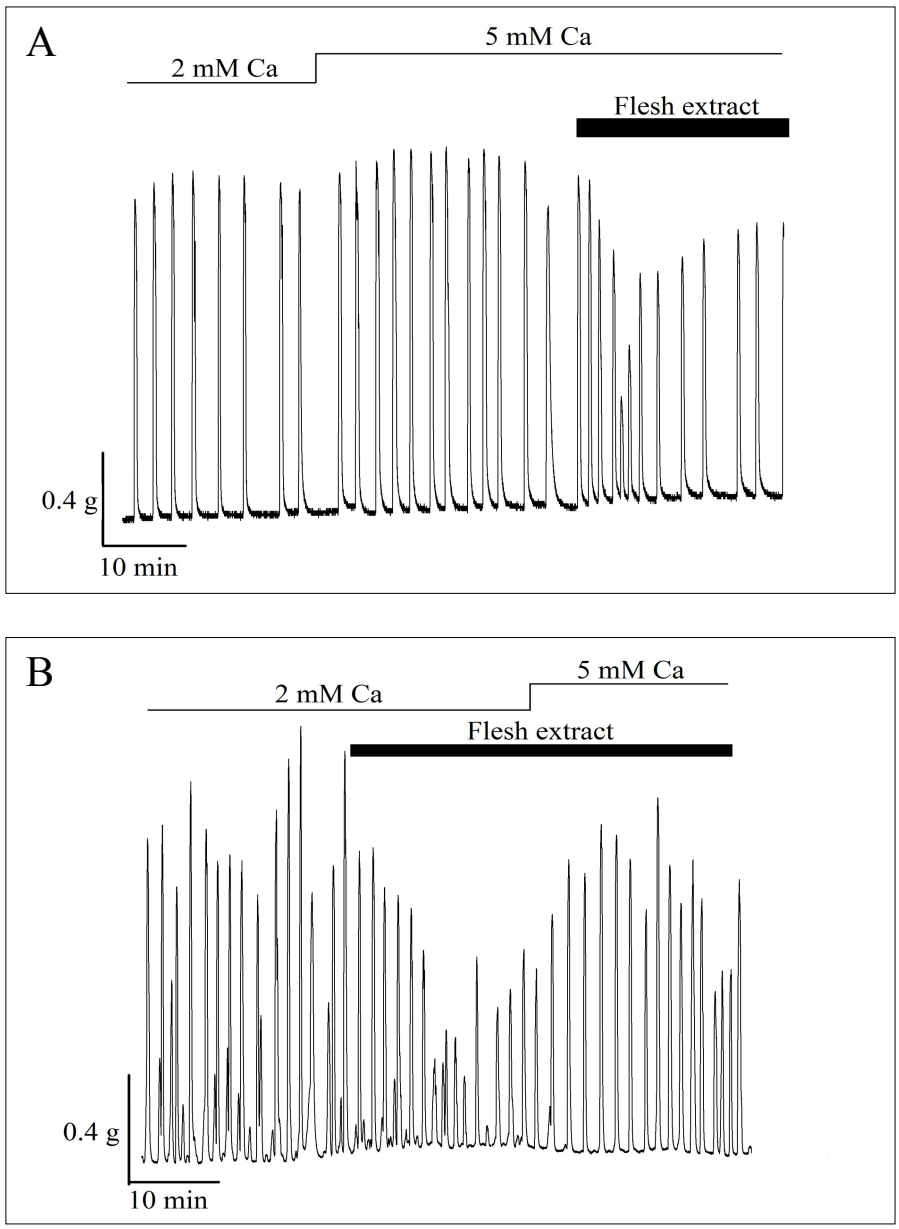

Figure 4.3 The effects of watermelon flesh extract on uterine contraction in the presence of high Ca^{2+}. 5 mM $CaCl_2$ solution was added before (A) and after (B) watermelon flesh extract (6 mg/mL; n = 5 for each).

Table 4.3 The effects of watermelon flesh extract on uterine contraction in the presence of high Ca^{2+}.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
Watermelon flesh (after)				
Control	100	100	100	5
5 mM $CaCl_2$	110.62 ± 1.52*	125.25 ± 2.51*	118.40 ± 0.95*	5
5 mM $CaCl_2$ + watermelon flesh	68.76 ± 3.40*	116.66 ± 1.09*	67.29 ± 2.93*	5
Watermelon flesh (before)				
Control	100	100	100	5
Watermelon flesh	67.02 ± 4.91*	114.00 ± 1.57*	70.02 ± 3.51*	5
Watermelon flesh + 5 mM $CaCl_2$	86.73 ± 2.71*	109.33 ± 4.80	85.80 ± 2.15*	5

The *P*-values for amplitude, frequency and AUC of 5 mM $CaCl_2$ treated are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

Watermelon Rind Extract

The inhibitory effects of rind extract on the response to high Ca^{2+} were also investigated. As can be seen in Figure 4.4A, high Ca^{2+} solution produced a significant increase in force. With high Ca^{2+}, rind extract significantly reduced the amplitude of contractions to 66.36 ± 3.66% ($P < 0.05$), compared to spontaneous contraction (100%, n = 5). Application of high Ca^{2+} solution in the continued presence of rind extract partially prevented the tocolytic effects of the rind extract (Figure 4.4B). The amplitude of force produced by the combination of rind extract and high Ca^{2+} solution measured after 10 minutes of addition was 86.20 ± 2.13% ($P < 0.05$), when compared with spontaneous contraction (100%, n = 5). The samples of experimental traces are shown in Figure 4.4 and data summarized in Table 4.4.

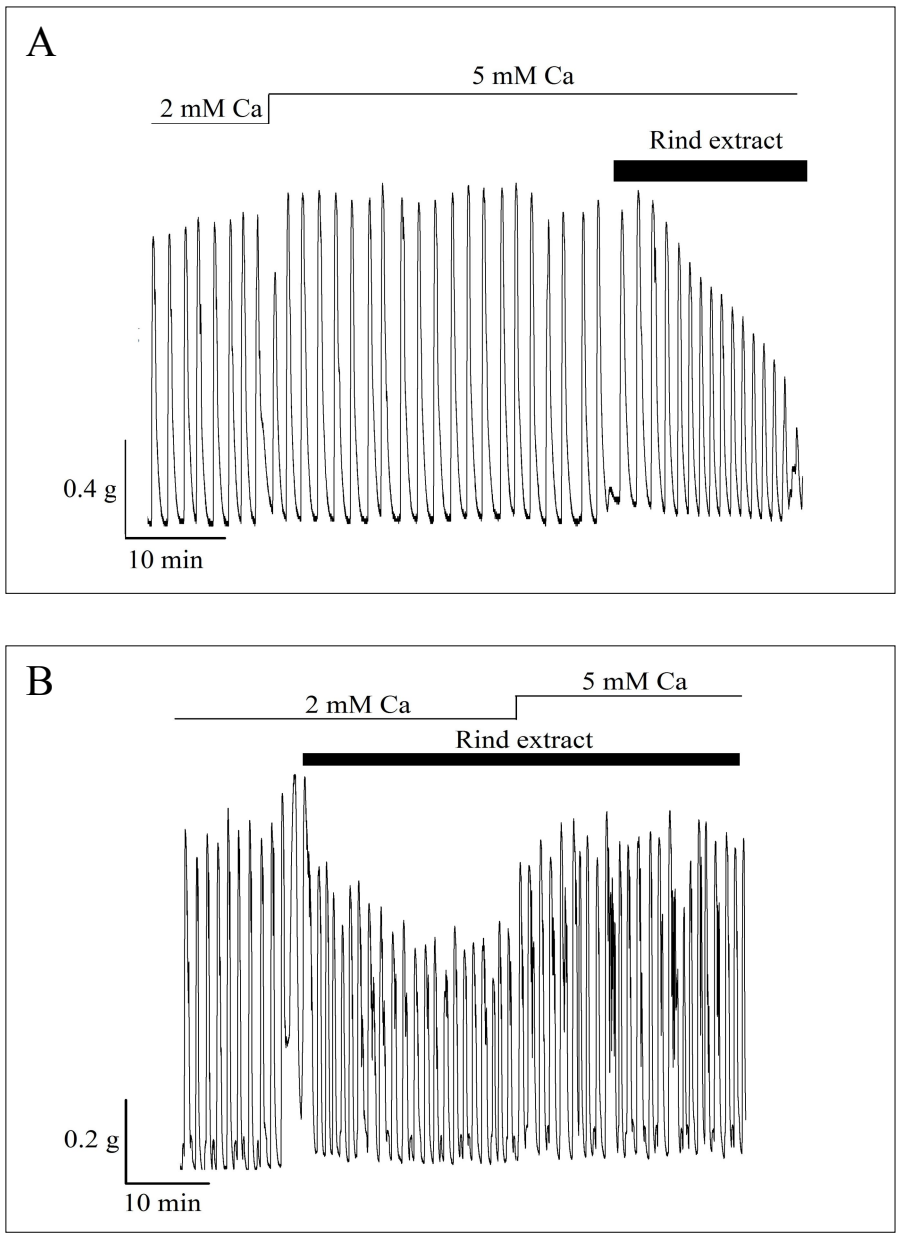

Figure 4.4 The effects of watermelon rind extract on uterine contraction in the presence of high Ca^{2+}. 5 mM $CaCl_2$ solution was added before (A) and after (B) watermelon rind (5 mg/mL) extract (n = 5).

Table 4.4 The effects of watermelon rind extract on uterine contraction in the presence of high Ca^{2+}.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
Watermelon rind (after)				
Control	100	100	100	5
5 mM $CaCl_2$	115.58 ± 4.11*	114.28 ± 3.84*	123.88 ± 8.09*	5
5 mM $CaCl_2$ + watermelon rind	66.36 ± 3.66*	114.63 ± 2.60*	67.29 ± 2.93*	5
Watermelon rind (before)				
Control	100	100	100	5
Watermelon rind	57.36 ± 7.62*	114.33 ± 3.66*	61.46 ± 5.00*	5
Watermelon rind + 5 mM $CaCl_2$	86.20 ± 2.13*	113.33 ± 1.33*	86.15 ± 2.48*	5

The *P*-values for amplitude, frequency and AUC of 5 mM $CaCl_2$ treated are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

4.4.3 Effects of Watermelon Flesh and Rind Extracts on $PGF_{2\alpha}$-Induced Uterine Contraction

Watermelon Flesh Extract

$PGF_{2\alpha}$ binds to prostaglandin FP receptor to stimulate uterine contractions through the activation of Ca^{2+} release from the SR (Hollingsworth, Downing, Cheuk, Piper and Hughes, 1993). As can be seen in Figure 4.5A, $PGF_{2\alpha}$ (1 μM) significantly increased the amplitude, the frequency and the mean integral force of the contractions. Application of flesh extract to the myometrial strips in the continued presence of $PGF_{2\alpha}$ produced a marked decrease in the amplitude and the mean integral force (n = 7). The samples of experimental traces are shown in Figure 4.5A and data summarized in Table 4.5.

Watermelon Rind Extract

As can be seen in Figure 4.5B, 1 μM $PGF_{2\alpha}$ significantly increased the amplitude, the frequency and the mean integral force of the contractions. Application of 5 mg/mL rind extract to the uterine strips in the continued presence of $PGF_{2\alpha}$ exerted significant inhibitory effects on both amplitude and the mean integral force (n = 7). The samples of experimental traces are shown in Figure 4.5B and data summarized in Table 4.5.

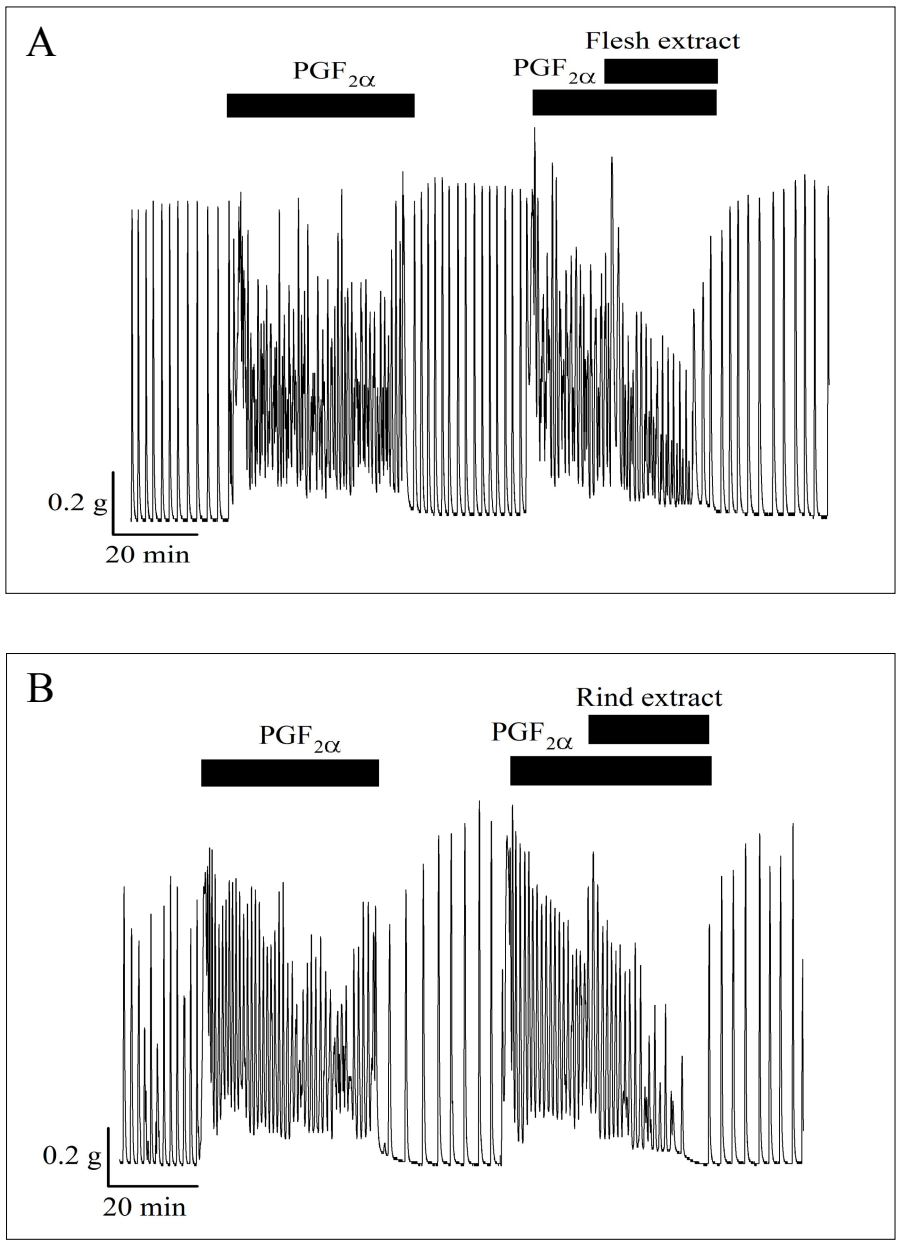

Figure 4.5 The effects of watermelon flesh and rind extracts on $PGF_{2\alpha}$-induced uterine contraction. The effects of 6 mg/mL flesh (A) and 5 mg/mL rind extracts (B) on uterine contraction-induced by 1 μM $PGF_{2\alpha}$ are shown (n = 7 for each).

Table 4.5 The effects of watermelon flesh and rind extracts on $PGF_{2\alpha}$-induced uterine contraction.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
Watermelon flesh				
Control	100	100	100	7
$PGF_{2\alpha}$	120.64 ± 0.91*	132.42 ± 0.83*	149.43 ± 8.53*	7
$PGF_{2\alpha}$ + watermelon flesh	60.37 ± 5.44*	114.46 ± 2.47*	65.80 ± 1.96*	7
Watermelon rind				
Control	100	100	100	7
$PGF_{2\alpha}$	127.03 ± 1.09*	130.32 ± 1.33*	140.85 ± 4.02*	7
$PGF_{2\alpha}$ + watermelon rind	55.74 ± 4.40*	116.66 ± 3.23*	54.78 ± 3.40*	7

The *P*-values for amplitude, frequency and AUC of 1 μM $PGF_{2\alpha}$ treated are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

4.4.4 Effects of Watermelon Flesh and Rind Extracts on Oxytocin-Induced Uterine Contraction

Watermelon Flesh Extract

Oxytocin is a nonapeptide hormone produced by the pituitary (Gimpl and Fahrenholz, 2001). It increases uterine force by acting on oxytocin receptor, which results in the Ca^{2+} influx through L-type Ca^{2+} channel and the release of Ca^{2+} from the SR (Gimpl and Fahrenholz, 2001; Phillippe and Chien, 1998; Shmygol, Gullam, Blanks and Thornton, 2006). As can be seen in Figure 4.6A, the addition of oxytocin (10 nM) significantly increased the amplitude, the frequency and the mean integral force of the contractions. Application of 6 mg/mL flesh extract to the myometrial strips in the continued presence of 10 nM oxytocin produced a decrease of force (n = 8). The samples of experimental traces are shown in Figure 4.6A and data summarized in Table 4.6.

Watermelon Rind Extract

As can be seen in Figure 4.6B, 10 nM oxytocin significantly increased contractile amplitude (110.59 ± 3.59%, $P < 0.05$) compared with control spontaneous contractions (100%, n = 8). Application of rind extract (5 mg/mL) to the myometrium in the continued presence of oxytocin caused a significant inhibition on both the amplitude and the mean integral force (n = 8). The samples of experimental traces are shown in Figure 4.6B and data summarized in Table 4.6.

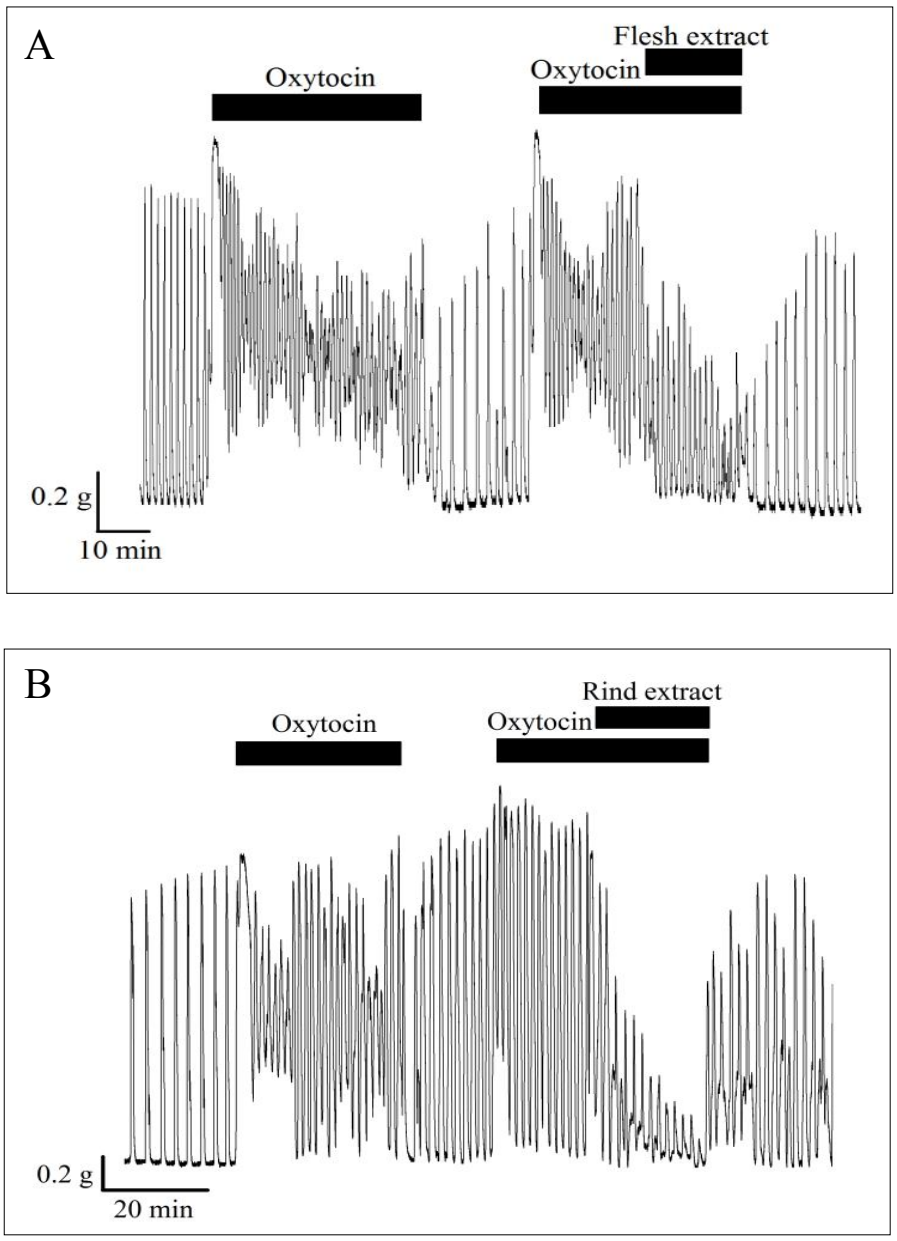

Figure 4.6 The effects of watermelon flesh and rind extracts on oxytocin-induced uterine contraction. The effects of 6 mg/mL flesh (A) and 5 mg/mL rind extracts (B) on uterine contraction-induced by 10 mM oxytocin are shown (n = 8 for each).

Table 4.6 The effects of watermelon flesh and rind extracts on oxytocin-induced uterine contraction.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
Watermelon flesh				
Control	100	100	100	8
Oxytocin	114.03 ± 3.61*	175.74 ± 6.31*	179.47 ± 7.49*	8
Oxytocin + watermelon flesh	55.39 ± 1.03*	116.73 ± 2.20*	50.19 ± 1.22*	8
Watermelon rind				
Control	100	100	100	8
Oxytocin	110.59 ± 3.59*	170.91 ± 2.21*	173.91 ± 3.52*	8
Oxytocin + watermelon rind	49.88 ± 9.45*	114.42 ± 1.75*	44.82 ± 6.43*	8

The *P*-values for amplitude, frequency and AUC of 10 nM oxytocin treated are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

4.4.5 Effects of Watermelon Flesh and Rind Extracts on KCl-Induced Uterine Contraction

Watermelon Flesh Extract

KCl solution-induced contraction is predominantly mediated by depolarization of the surface membrane and Ca^{2+} entry through voltage-dependent Ca^{2+} channels (Ausina, Savineau, Pinto, Martin and Candenas, 1996). As can be seen in Figure 4.7A, the addition of KCl produced an initial rapid phasic contraction followed by a sustained tonic contraction. Upon return to control solution, uterine tension reappeared after a delay of approximately 5 min. 30 min later, the solution was changed to KCl again. This resulted initially in a rise of tension to approximately the same level as seen with the first treatment of KCl. The flesh extract (6 mg/mL) was added to the myometrium during the sustained tonic contraction. It produced a significant inhibition of the contraction (n = 6). This value was 73.19 ± 4.67% ($P < 0.05$) compared to the KCl control (100%).

Watermelon Rind Extract

As can be seen in Figure 4.7B, isoosmotic KCl (40 mM) solution significantly increased uterine force. The contraction was maintained as long as KCl was present. Application of 5 mg/mL rind extract to the myometrium in the continued presence of KCl produced a significant inhibition on force (n = 6). This value was 56.12 ± 1.25% ($P < 0.05$) compared to the KCl control (100%).

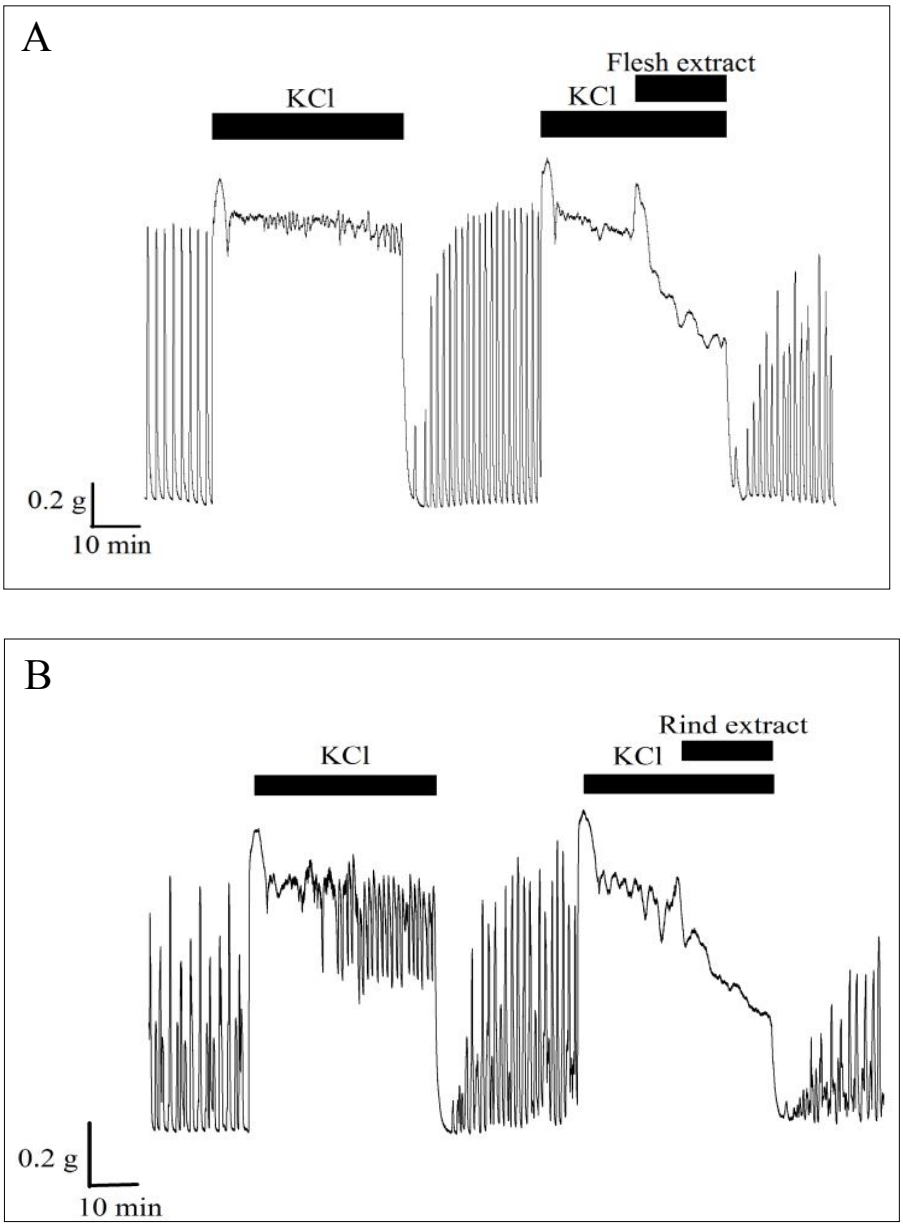

Figure 4.7 The effects of watermelon flesh and rind extracts on KCl-induced uterine contraction. The effects of 6 mg/mL watermelon flesh (A) and 5 mg/mL rind extracts (B) on uterine contraction-induced by KCl (40 mM) solution are shown (n = 6 for each).

4.4.6 Effects of Watermelon Flesh and Rind Extracts on $PGF_{2\alpha}$-Induced Uterine Contraction in the Absence of External Ca^{2+}

Watermelon Flesh Extract

It is well known that in the absence of external Ca^{2+}, force was rapidly abolished (Buddhakala et al., 2008; Matthew, Kupittayanant, Burdyga and Wray, 2004; Kupittayanant et al., 2002). Under this condition, some agonists can generate the contractile force through the release of Ca^{2+} from the SR (Matthew et al., 2004; Kupittayanant et al., 2002; Wray et al., 2003). Thus, it was of interest to investigate whether the flesh extract could alter this contraction. $PGF_{2\alpha}$ was added in the absence of external Ca^{2+} entry. This protocol was performed to ensure that the only source of Ca^{2+} was from the SR (Matthew et al., 2004; Kupittayanant et al., 2002). As can be seen in Figure 4.8A, phasic contractions abolished upon changing to 0-Ca solution. In the continued presence of 0-Ca solution, 1 μM $PGF_{2\alpha}$ produced a small tonic force as long as this agonist was present. Upon return to control solution, spontaneous phasic contractions reappeared. 30 min later, the solution was changed to one containing 0-Ca and flesh extract (6 mg/mL). Force abolished rapidly and $PGF_{2\alpha}$ added. It was revealed that very little if any force was produced (Figure 4.8A, n = 6). This value was 30.35 ± 5.25% ($P < 0.05$) compared to $PGF_{2\alpha}$-induced contraction in the absence of external Ca^{2+} alone (100 %, n = 6).

Watermelon Rind Extract

The effects of watermelon rind extract on $PGF_{2\alpha}$-induced uterine contraction in the absence of external Ca^{2+} were also examined. The effects were similarly to the effects of flesh extract. As can be seen in Figure 4.8B, $PGF_{2\alpha}$ produced a small tonic force in 0-Ca solution. It was found that watermelon rind extract impeded this contraction to 25.42 ± 4.13% ($P < 0.05$, n = 6) compared to $PGF_{2\alpha}$-induced contraction in the absence of external Ca^{2+} alone (100 %, n = 6).

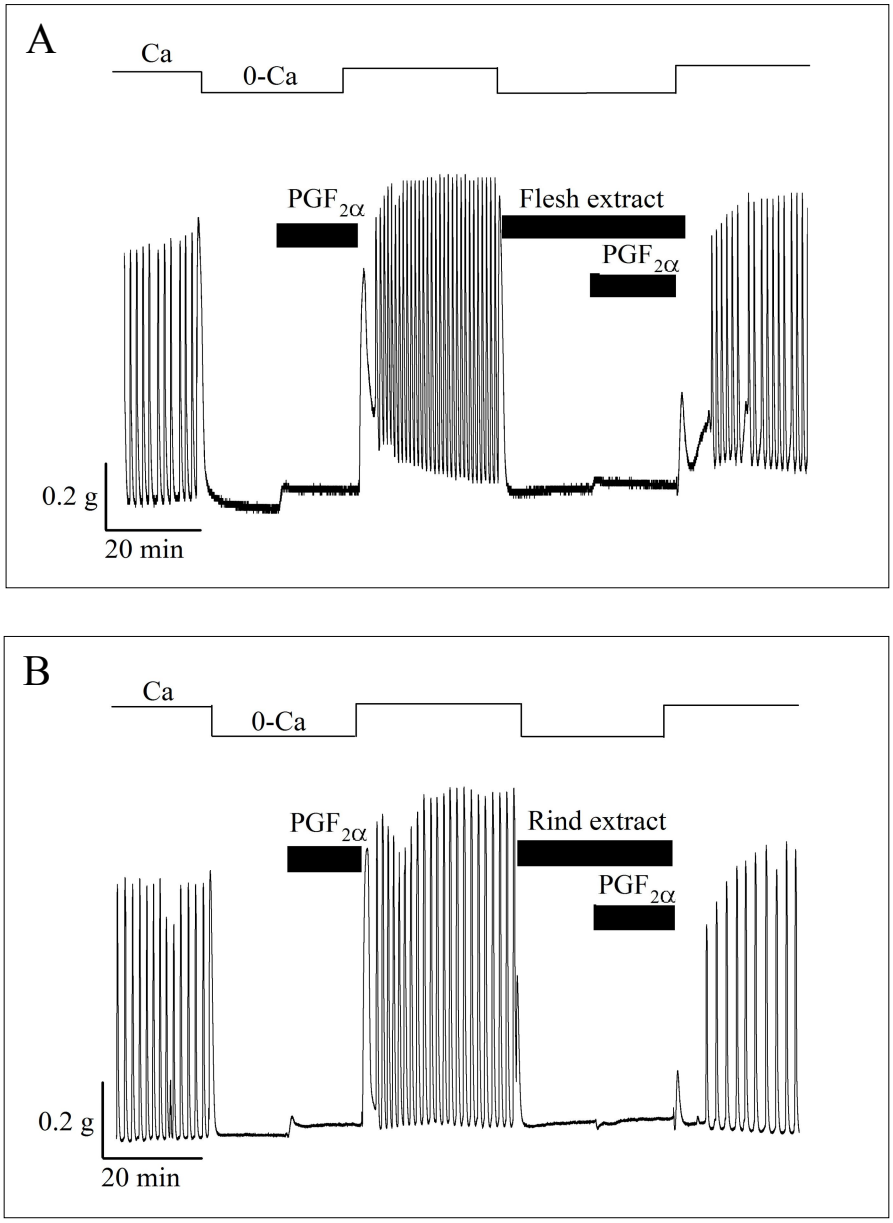

Figure 4.8 The effects of watermelon flesh and rind extracts on $PGF_{2\alpha}$-induced uterine contraction in the absence of external Ca^{2+}. The effects of 6 mg/mL watermelon flesh (A) and 5 mg/mL rind extracts (B) on uterine contraction-induced by 1 μM $PGF_{2\alpha}$ in 0-Ca solution are shown (n = 6 for each).

4.4.7 Effects of Watermelon Flesh and Rind Extracts on Oxytocin-Induced Uterine Contraction in the Absence of External Ca^{2+}

Watermelon Flesh Extract

The same protocol was used as described above for $PGF_{2\alpha}$. In the absence of external Ca^{2+}, 10 nM oxytocin produced a small tonic force, indicating that oxytocin can release Ca^{2+} from the SR (Matthew et al., 2004; Kupittayanant et al., 2002). As can be seen in Figure 4.9A, application of 6 mg/mL watermelon flesh extract to the uterine strips caused a significant inhibition of the contraction induced by oxytocin in the absence of external Ca^{2+}. This value was 35.63 ± 2.52% ($P < 0.05$, n = 6) compared to oxytocin-induced contraction in the absence of external Ca^{2+} alone (100 %, n = 6).

Watermelon Rind Extract

As can be seen in Figure 4.9B, application of watermelon rind extract (5 mg/mL) to uterine contraction-induced by oxytocin in the absence of external Ca^{2+} produced a marked significant inhibition of the contraction. This value was 30.83 ± 5.18% ($P < 0.05$, n = 6) compared to oxytocin-induced contraction in the absence of external Ca^{2+} alone (100 %, n = 6).

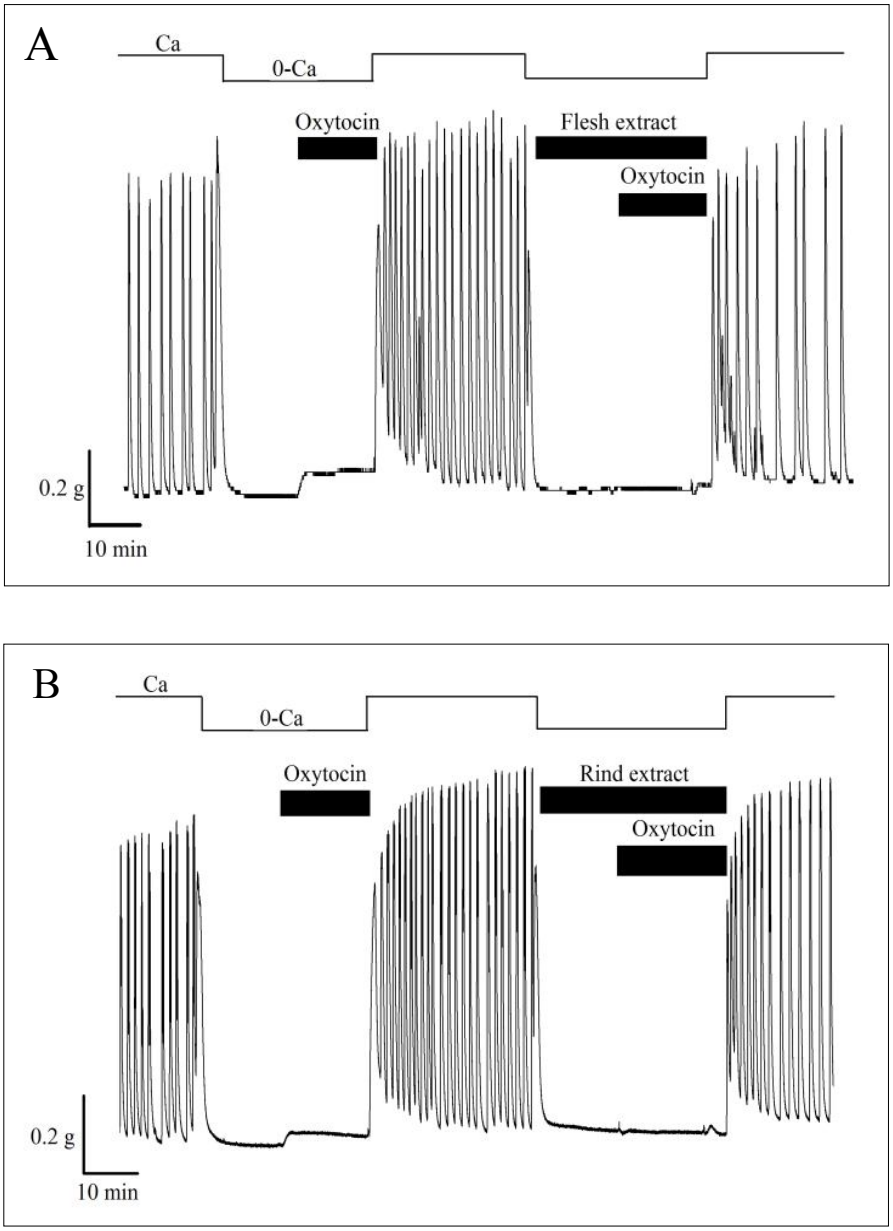

Figure 4.9 The effects of watermelon flesh and rind extracts on oxytocin-induced uterine contraction in the absence of external Ca^{2+}. The effects of 6 mg/mL watermelon flesh (A) and 5 mg/mL rind extracts (B) on uterine contraction-induced by 10 nM oxytocin in 0-Ca solution are shown (n = 6 for each).

4.4.8 Effects of Watermelon Flesh and Rind Extracts on Oxytocin-Induced Uterine Contraction in the Presence of KCl

Watermelon Flesh Extract

It has been reported that, when Ca^{2+} was high or maintained, smooth muscle contraction may be modulated by Ca^{2+}-independent pathway. For example, application of oxytocin in the presence of KCl generated a tonic component of force, indicating the release of Ca^{2+} from the SR. In addition, it was demonstrated that the production of force by oxytocin may due to the modulation of myosin light chain phosphatase (MLCP) activity through rho-associated kinase (ROK) pathway (Kupittayanant, Burdyga and Wray, 2001; Somlyo and Somlyo, 1998). ROK stimulated by oxytocin produced a marked increase in force without a change in intracellular Ca^{2+} (Kupittayanant et al., 2001). It was of interest to verify whether watermelon flesh extract may alter this kind of contraction; oxytocin-induced uterine contraction in the presence of KCl. As can be seen in Figure 4.10A, application of oxytocin (10 nM) in the continued presence of KCl produced a small tonic contraction, suggesting the release of Ca^{2+} from the SR. Upon return to control solution, uterine tone quickly returned to baseline and spontaneous phasic contractions reappeared. 30 min later, the solution was then changed to KCl and oxytocin added. In addition, 6 mg/mL flesh extract was added to the uterine strip during the sustained tonic contraction. It produced a marked decrease in force. Thus, after 10 min, force had fallen to 74.98 ± 3.80% of control force development ($P < 0.05$, 100% force was the level of maintained force that was produced during the application of 10 nM oxytocin in the presence of KCl, n = 6).

Watermelon Rind Extract

The same protocol was used as described above for the flesh extract. As can be seen in Figure 4.10B, application of 5 mg/mL rind extract was able to decrease force upon oxytocin application to the uterus in the presence of KCl. After 10 min, force had fallen to 73.81 ± 1.59% of control force development ($P < 0.05$, 100% force was the level of maintained force that was produced during the application of 10 nM oxytocin in the presence of KCl, n = 6).

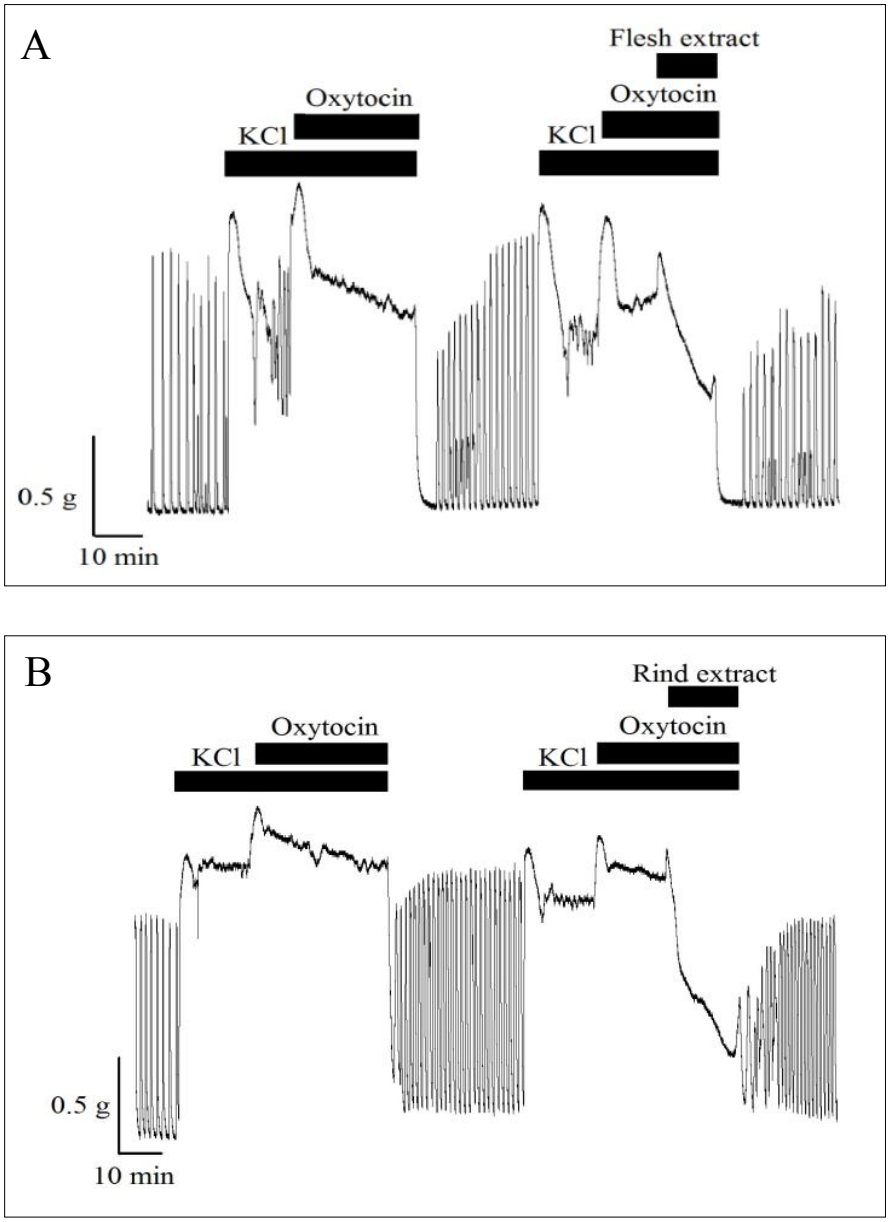

Figure 4.10 The effects of watermelon flesh and rind extracts on oxytocin-induced uterine contraction in the presence of KCl. The effects of 6 mg/mL watermelon flesh (A) and 5 mg/mL rind extracts (B) on oxytocin-induced uterine contraction in the presence of 40 mM KCl solution are shown (n = 6 for each).

4.5 Discussion

To the best of our knowledge, this study is the first to demonstrate the effects of watermelon extracts on agonists-induced uterine contractions. The prominent effects of watermelon on force partially mediate through the inhibition of L-type Ca^{2+} channels. In addition, watermelon flesh and rind extracts potentially inhibit uterine contractions-induced by $PGF_{2\alpha}$, oxytocin, and KCl. In the absence of external Ca^{2+}, both watermelon flesh and rind extracts impede the contraction-induced by $PGF_{2\alpha}$ and oxytocin. In addition, they caused a marked decrease in tonic contractions produced by oxytocin-induced contraction in the presence of KCl. The relaxation of force produced by the extracts was dependent upon (1) Ca^{2+}-dependent, (2) Ca^{2+}-independent regulation of smooth muscle contraction as well as, (3) NO-cGMP pathway modulation.

It is generally accepted that in uterine smooth muscle cells, a rise of $[Ca^{2+}]_i$ plays a crucial role in the mechanism of contraction. Ca^{2+} for the contraction is due to the release of the SR and the influx of extracellular Ca^{2+} through the L-type Ca^{2+} channels (Matthew et al., 2004; Kupittayanant et al., 2002). The increased in $[Ca^{2+}]_i$ results in calmodulin activation of myosin light chain kinase. This kinase phosphorylates the regulatory light chains of myosin, leading to the activation of myosin MgATPase and force generation and/or shortening of the muscle fibers. (Kupittayanant et al., 2002; Phillippe and Edward, 1998; Wray, 1993; 2007; Wray et al., 2003).

Bay K8644 was used to activate the L-type Ca^{2+} channels (Chien et al., 1996). Opening of cell membrane Ca^{2+} channels mediates the influx of Ca^{2+} ion to

generate force (Chien et al., 1996). These present studies were performed to test the hypothesis that Bay K8644 can inhibit or prevent the inhibitory effects of watermelon extracts on rat uterine contraction. Application of Bay K8644 (1 μM) to spontaneous contractions produced an increase in the amplitude, the frequency, and the mean integral force of phasic contractions when compared with the control period. Watermelon flesh (6 mg/mL) or rind (5 mg/mL) extract partially inhibited uterine contractions induced by Bay K8644. Thus, this finding suggests that watermelon extracts partially inhibit force production through the interruption of extracellular Ca^{2+} influx. In addition, it also indicates that other mechanisms could take part in the tocolytic effects of watermelon extracts on uterine contraction (see below).

In other set of experiments, application of high Ca^{2+} was also used to investigate the effects of watermelon extracts on L-type Ca^{2+} channels (Buddhakala et al., 2008). Similarly, in Bay K8644, the addition of 5 mM $CaCl_2$ solution to the organ bath caused a significant increase in contractile activity. Application of watermelon extracts suppressed uterine contraction-induced by 5 mM $CaCl_2$, suggesting that the tocolytic effects of watermelon rat uterine contraction may partially be due to the inhibition of Ca^{2+} entry through L-type Ca^{2+} channels (Buddhakala et al., 2008).

Exposure of the uterine strips to KCl induces a rapid rise in $[Ca^{2+}]_i$ through depolarization of the cell membrane, leading to the opening of L-type Ca^{2+} channels and muscle contraction (Ausina et al., 1996; Kupittayanant et al., 2001). This tonic contraction is diminished by some agents that interfere at the levels of excitation-contraction coupling, such as Ca^{2+} channel blocking agents (Ausina et al., 1996; Ivorra, Chuliá, Noguera and D'Ocon, 1994). The addition of watermelon extracts to

the uterus was able to decrease force in the presence of KCl, supporting the hypothesis that watermelon extracts possess a Ca^{2+} entry blocking activity.

$PGF_{2\alpha}$ and oxytocin increase uterine contractile activity by acting on prostaglandin FP and oxytocin receptors, respectively (Gimpl and Fahrenholz, 2001; Phillippe and Edward, 1998; Soloff, 1990). These receptors were connected with $G\alpha_{q/11}$ subunit to membrane phospholypase Cβ (PLCβ). Hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) produces 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 binds to its receptor on the SR membrane and causes release of Ca^{2+} into the cytoplasm (Gimpl and Fahrenholz, 2001; Phillippe and Edward, 1998; Soloff, 1990). Moreover, oxytocin itself can activate L-type Ca^{2+} channels and chloride (Cl_{Ca}) channels directly, resulting in the increase in phasic contractions (Arnaudeau, Lepretre and Mironneau, 1994; Wray et al., 2003). The application of $PGF_{2\alpha}$ and oxytocin to the uterine strips produced a marked increase of the contractile force. The addition of watermelon flesh or rind extract in the presence of $PGF_{2\alpha}$ or oxytocin decreased uterine contractility. Thus, these findings suggest that the spasmolytic activity of watermelon extracts may be due to the interruption of $G\alpha_{q/11}$-PLCβ-IP_3 pathway and the decrease of Ca^{2+} entry.

The mechanisms of action by which natural products produce tocolytic effects are also associated with the NO system (Drewes, George and Khan, 2003; Jayaprakasha et al., 2011). It has been reported that NO induces changes in target proteins through the activation of cGMP (Drewes et al., 2003; Jayaprakasha et al., 2011). There is evidence that watermelon extract can decrease the levels of $[Ca^{2+}]_i$ by using Flou-4 AM dye, a specific fluorescent probe for $[Ca^{2+}]_i$ in smooth muscle cell line (Jayaprakasha et al., 2011). It has been hypothesized that the mechanisms

underlying of NO and cGMP could affects $[Ca^{2+}]_i$ in four different ways: 1) by reducing Ca^{2+}; 2) by increasing Ca^{2+}efflux; 3) by enhancing Ca^{2+} sequestration; and 4) by decreasing Ca^{2+} mobilization (Lincoln, Cornwell, Komallavilas, Macmillan-Crow and Boerth, 1995; McDonald and Murad, 1995; Norman, 1996). Based on these scientific evidences, it is possible to speculate that NO-cGMP pathway modulation may also be involved in the inhibitory effects of watermelon flesh and rind extracts on rat uterine contraction in this present study.

In 0-Ca solution, $PGF_{2\alpha}$ and oxytocin can elicit a small tonic contraction, suggesting the release of Ca^{2+} from the SR. Pre-incubation of watermelon extracts with 0-Ca solution altered the contraction triggered by $PGF_{2\alpha}$ or oxytocin. It has been demonstrated that cGMP-dependent protein kinase (PKG Iβ) can decrease IP_3-induced elevations in $[Ca^{2+}]_i$ through the phosphorylation of the IP_3R-associated cGMP kinase substrate (IRAG) (Schlossmann et al., 2000). IRAG was found in many tissues, including the uterus (Schlossmann et al., 2000). It is indicated that IRAG may be associated with the dynamics of Ca^{2+} release by either by modulating IP_3R function or by regulating mechanisms that provide electroneutrality of Ca^{2+} release (Nguyen, Chin and Verdugo, 1998). Co-expression of IRAG and PKG Iβ in the presence of cGMP inhibited the release of Ca^{2+} induced by bradykinin (Schlossmann et al., 2000). Based on these scientific data, it is possible to assume that watermelon extracts affected a reduction on the SR release by interrupting $G\alpha_{q/11}$-PLCβ-IP_3 pathway.

It has been demonstrated that, when Ca^{2+} was high or maintained, smooth muscle contraction may be modulated by Ca^{2+}-independent pathway (Kupittayanant et al., 2001). For example, application of oxytocin in the presence of KCl produced a tonic component of force, indicating release of Ca^{2+} from the SR. In addition, it was

suggested that oxytocin may modulate of MLCP activity through the ROK pathway to increase the contractile force (Kupittayanant et al., 2001; Shmygol et al., 2006; Somlyo and Somlyo, 1998). ROK activated by oxytocin produced a marked increase in force without a change in $[Ca^{2+}]_i$ (Kupittayanant et al., 2001). In this prevalence, the application of watermelon extracts to oxytocin-induced contraction in the presence of KCl produced a marked decrease in uterine force. This finding indicates that watermelon extracts might inhibit, at least in part, the ROK pathway induced a Ca^{2+} desensitization.

There is a significant clinical need for uterine relaxants in the treatment of primary dysmenorrhea and preterm labor (Gruber and O'Brien, 2011; Hollingsworth et al., 1993). However, etiologies of these disorders are unknown because they are influenced by many aspects (Hollingsworth et al., 1993). Thus, drugs could achieve tocolytic effects by several mechanisms. It is well known that medicinal plants contain various phytochemical constituents that exert their effects through multi-cellular targets in the body (Kunnumakkara et al., 2009). This could be the reason why the medicinal plants have effective in the treatment of various diseases. The results of this present study indicate that watermelon extracts produce a tocolytic effect on uterine contractions-induced by $PGF_{2\alpha}$ and oxytocin. Therefore, these findings provide scientific evidence supporting the therapeutic uses of watermelon in the treatment of primary dysmenorrhea and preterm labor.

In conclusion, this study investigated the inhibitory effects of watermelon extracts on isolated rat uterus. The results indicate that watermelon flesh and rind extracts produced tocolytic effects by inhibiting both Ca^{2+}-dependent and Ca^{2+}-independent regulation of smooth muscle contraction pathways as well as by

stimulating NO-cGMP pathway modulation. It is suggested that watermelon has the spasmolytic potency for the prevention of dysmenorrhea and preterm labour.

4.6 References

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CHAPTER V

EFFECTS OF L-CITRULLINE AND L-ARGININE ON SPONTANEOUS AND AGONISTS-INDUCED UTERINE CONTRACTIONS

5.1 Abstract

Watermelon (*Citrullus lanatus*) is rich in L-citrulline and L-arginine; the contents that play an important role in the production of the potent vasodilator, nitric oxide (NO). As shown in the previous chapters, watermelon extracts seem to have tocolytic effects as they inhibited uterine contraction. Thus, the present study was designed to investigate whether the tocolytic effects of watermelon extracts was due to those of the potent vasodilators; L-citrulline and L-arginine. The contractile responses in the presence of L-citrulline and L-arginine were recorded isometrically with a force transducer. The results showed that L-citrulline and L-arginine (1×10^{-6} to 1×10^{-3} M) had a dose-dependent effect on spontaneous contraction. The EC_{50} values of L-citrulline and L-arginine were 64 μM and 104 μM, respectively. In addition, L-citrulline and L-arginine impeded uterine contractions induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), oxytocin, and potassium chloride solution (KCl). They also reduced $PGF_{2\alpha}$- and oxytocin-induced uterine force in the absence of external calcium (Ca^{2+}) and partially inhibited contraction induced by increasing external

Ca^{2+}. In addition, they caused a marked decrease in tonic contractions produced by oxytocin-induced contraction in the presence of KCl. These data indicated that the tocolytic effects of L-citrulline and L-arginine were via the inhibition of both Ca^{2+}-dependent and Ca^{2+}-independent pathways of force regulation. The effects of L-citrulline and L-arginine were similar to those produced by watermelon extracts. Thus, the results suggest that the inhibitory effects of watermelon could be due to L-citrulline and L-arginine contents.

5.2 Introduction

Fruit and vegetable consumption has been associated with reduced incidence of some chronic diseases such as atherosclerosis and cancers. Watermelon is rich in phytoneutrients such as lycopene, which is a well known antioxidant and has a potential role in prevention of prostate cancer (Perkins-Veazie, Maness and Roduner, 2002). Watermelon is also an excellent source of vitamins and minerals. It has been reported that watermelon contains a high level of amino acids, L-citrulline and L-arginine (Tlili, Hdider, Lenucci, Ilahy, Jebari and Dalessandro, 2011). These two amino acids play a crucial role in NO system by eliciting a wide range of such as vasorelaxation, vascular functions, anti-inflammatory, and anti-platelet activity (Flam, Hartmann, Harrell-Booth, Solomonson and Eichler, 2001; Norman, 1996).

5.2.1 L-Citrulline

L-citrulline ($C_6H_{13}N_3O_3$) is water soluble α-amino acid with an asymmetric carbon. It is a natural precursor for L-arginine to produce NO (Lincoln, Cornwell, Komallavilas, Macmillan-Crow and Boerth, 1995; Romero, Platt, Caldwell and Caldwell, 2006). Thus, under pathological conditions, L-citrulline may relatively substitute for L-arginine. L-citrulline can be converted to L-arginine in all cell types (Flam et al., 2001; Romero et al., 2006). It was shown that L-citrulline plays an important role in providing L-arginine to NO synthase (NOS) as it bypasses metabolism in the liver (Flam et al., 2001; Romero et al., 2006). The normal range of plasma levels of L-citrulline in humans is 22.9 - 65 μM (Ochiai et al., 2010). In recent decades, little scientific evidence has been reported the use of L-citrulline as the therapeutic agent (Cormio et al., 2011; Ochiai et al., 2010; Romero et al., 2006). As mentioned above, L-citrulline can be used to substitute and/or convert to L-arginine. Thus, L-citrulline may be served as a new important substance for new treatments (Cynober, Moinard and De Bandt, 2010; Flam et al., 2001).

It has been suggested that short-term L-citrulline supplementation can improve arterial stiffness in humans (Ochiai et al., 2010). It was indicated that oral L-citrulline supplementation can attenuate brachial blood pressure and aortic pulse pressure responses to cold presser test in young men (Figueroa, Sanchez-Gonzalez, Perkins-Veazie and Arjmandi, 2010). Ranghavan and Dikshit (2001) was undertaken to investigate the relaxant effects of L-citrulline mediated relaxation in the lipopolysaccharide (1 mg/kg)-treated rat aortic rings. The results showed that L-citrulline produced 40 ± 3% and 60 ± 5% relaxant in control and lipopolysaccha-ride-treated rats, respectively. The relaxant effects of L-citrulline in rabbit vascular

smooth muscle were also reported (Ruiz and Tejerina, 1998). This study indicated that L-citrulline mediated vascular relaxation thought the production of cGMP mediated by NO and the opening of calcium-activated potassium (K_{Ca}) channels (Ruiz and Tejerina, 1998).

5.2.2 L-Arginine

L-arginine ($C_6H_{14}N_4O_2$) is a basic amino acid in physiological fluids. L-arginine is involved in various metabolic pathways, such as synthesis of creatinine, L-ornithine, L-glutamate, and polyamines (Norman, 1996). L-arginine is also involved in NO production by serving as the substrate of a family of NOS (Norman, 1996). It is well known that NO plays a critical role in regulating the function of many organs throughout the body (Norman, 1996). The normal range of plasma L-arginine levels in healthy volunteers is 49 - 236 μM (Romero et al., 2006).

It has been found that L-arginine supplementation can improve reproductive, cardiovascular, pulmonary, renal, gastrointestinal, liver, and immune functions (Flynn, Meininger, Haynes and Wu, 2002; Racké and Warnken, 2010). Thus, L-arginine may provide novel and effective therapies for obesity, diabetes, and the metabolic syndrome (Racké and Warnken, 2010). L-arginine is associated with endothelial dysfunction in preeclamptic women. It is indicated that L-arginine levels and placental endothelial NOS abundance are decreased in preeclamptic compared to healthy pregnant women (Roberts, 1999). Infusion of L-arginine at the dose of 30 g over 30 min into women with the preterm labour can decrease spontaneous uterine contractility (Facchinetti, Saade and Neri, 2007). It has been reported that L-arginine 0.01 mM caused a significant decrease in the uterine contractility (Hoffmann, Stanke-

Labesque, Fanchin, Dilaï, Ponsand and Ayoubi, 2003). However, pre-treatment the myometrial strip with methylene blue, a soluble guanyltate cyclase inhibitor, can prevent the inhibitory effects of L-arginine. In addition, L-NAME, a non-specific NOS synthase inhibitor, significantly inhibited the L-arginine-induced relaxation (Hoffmann et al., 2003).

There are number of compounds found in watermelon (Rimando and Perkins-Veazie, 2005; Tlili et al., 2011). Based on epidemiological and clinical data, it was of interest to investigate whether the tocolytic effects of watermelon may be due to L-citrulline and L-arginine. Thus, the aim of this chapter was to examine the effects of L-citrulline and L-arginine on rat uterine contractions based on Ca^{2+}-dependent and Ca^{2+}-independent regulation of smooth muscle contraction.

5.3 Materials and Methods

5.3.1 Myometrial Tissue Preparations and Measurements of Tension

Myometrial tissue preparations were dissected and force measurements measured as those described in Chapter II (Sections 2.2.3 and 2.2.4, respectively).

5.3.2 Experimental Procedures

5.3.2.1 Dose Dependency of L-Citrulline and L-Arginine

Following a 30-min equilibration time, L-citrulline or L-arginine was added into the bathing solution in a cumulative increase in concentration manner. Concentration-response curves were plotted and calculated the median effective

concentrations (EC_{50} values, concentration required to produce 50% of maximum inhibition of the amplitude of contraction) by a nonlinear curves fitting program (Vergara-Galicia et al., 2010). The EC_{50} values of each agent were applied throughout the experiments.

5.3.2.2 Effects on Spontaneous Uterine Contraction

Following a 30-min equilibration time, the EC_{50} values of L-citrulline or L-arginine were added into the bathing solution and incubated for 30 min. At the end of the experiment, the bathing solution was replaced by Krebs' solution and tension monitored up to 30 min.

5.3.2.3 Effects on Bay K8644- and Increasing $CaCl_2$ Concentration-Induced Uterine Contractions

To investigate whether the effects of L-citrulline and L-arginine were dependent upon external Ca^{2+}entry through voltage-gated L-type Ca^{2+} channels, Bay K8644, an L-type Ca^{2+} agonist, or increasing $CaCl_2$ concentration up to 5 mM was used to induce uterine contraction and the effects of L-citrulline and L-arginine studied. To do so, Bay K8644 or 5 mM $CaCl_2$ was applied for 30 min and then the extracts applied, in the continued presence of Bay K8644 or 5 mM $CaCl_2$. In addition, the experiments were done the other way round. To do so, L-citrulline or L-arginine was applied for 30 min and then Bay K8644 or 5 mM $CaCl_2$ added, in the continued presence of Bay K8644 or 5 mM $CaCl_2$.

5.3.2.4 Effects on $PGF_{2\alpha}$-, Oxytocin- and KCl-Induced Uterine Contractions

The effects of L-citrulline and L-arginine on $PGF_{2\alpha}$-, oxytocin- and KCl-induced uterine contractions were evaluated as follows. After equilibration period in Krebs' solution, the uterine strip was stimulated with $PGF_{2\alpha}$ (1 μM), oxytocin (10 nM) or KCl (40 mM) for 40 min and then washed. 30 min later $PGF_{2\alpha}$, oxytocin or KCl was then added into the bathing solution, and 20 min later L-citrulline or L-arginine was incubated for 20 min in the continued presence of agonists or KCl. At the end of the experiment, the bathing solution was replaced by Krebs' solution and tension monitored up to 30 min. The results of these experiments were compared with the contraction without L-citrulline or L-arginine.

5.3.2.5 Effects on $PGF_{2\alpha}$- and Oxytocin-Induced Uterine Contractions in the Absence of External Ca^{2+}

The uterine strip was incubated with 0-Ca (1 mM EGTA) solution containing L-citrulline or L-arginine during 15 min, then 1 μM $PGF_{2\alpha}$ or 10 nM oxytocin was added to stimulate the release of Ca^{2+} from the SR. The maximal tension produced by $PGF_{2\alpha}$ or oxytocin in the control group (without the L-citrulline or L-arginine) was considered as 100%.

5.3.2.6 Effects on Oxytocin-Induced Uterine Contractions in the Presence of KCl

The uterine strip was incubated with KCl for 15 min. Then the solution in the bath was replaced by KCl containing 10 nM oxytocin and equilibrated for 15 min. After the maximum contractile response to KCl containing oxytocin was obtained, L-citrulline or L-arginine was then applied for 15 min. At the end of the experiment, the bathing solution was replaced by Krebs' solution and tension monitored up to 30 min.

5.3.3 Chemicals and Physiological Solutions

All chemicals were purchased from Sigma®, Singapore. L-citrulline and L-arginine were dissolved in Krebs' solution just before use. Bay K8644, (the L-type Ca^{2+} channel activator; S-(−)-1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)-phenyl]-3-pyridine-carboxylic acid methyl ester), was dissolved in absolute ethanol and used at the concentration of 1 μM (Kupittayanant, Kupittayanant and Suwannachat, 2008). A 5 mM $CaCl_2$ solution was made by increasing the concentration of Ca^{2+} ($CaCl_2$) from 2 to 5 mM into Krebs' solution (Buddhakala, Talubmook, Sriyotha, Wray and Kupittayanant, 2008). The agonist oxytocin was dissolved in distilled water and used at concentration of 10 nM to produce a phasic contraction (Kupittayanant, Luckas and Wray, 2002). Prostaglandin $F_{2\alpha}$, ($PGF_{2\alpha}$-tris; (5Z,9$_\alpha$,11$_\alpha$,13E,15)-9,11,15-trihydroxyprosta-5,13-dienoic acid tris salt), was dissolved in the absolute ethanol and used at the concentration of 1 μM (Buddhakala et al., 2008). A KCl (40 mM) Krebs' solution was made by isoosmotic replacement of

sodium chloride (Noble and Wray, 2002). In some experiments, 0-Ca solutions were used; physiological solution in which $CaCl_2$ had been omitted and ethylene glycol tetraacetic acid (EGTA, 1 mM) added (Kupittayanant et al., 2002). The physiological Krebs' solution (pH 7.4) contained the following (mM): NaCl 154.0, KCl 5.4, $MgSO_4$ 1.2, glucose 8.0, $CaCl_2$ 2.0, and N-2-hydroxyethylpiperazine-N'-2-ethanesul-fonic acid (HEPES) 10.0.

5.3.4 Statistical Analysis

The data were analyzed using Microcal Origin Software. The integral force was used as the parameter of contraction. Results were expressed as percentages of control contractions (i.e. the control is 100%). Throughout, data are presented as mean ± S.E.M. and "n" represents the number of samples, each one from a different animal. Significances were tested using appropriate *t* tests. The *P* value < 0.05 was taken to be significant.

5.4 Results

5.4.1 Dose Dependency of L-Citrulline

The effects of increasing cumulative concentrations of L-citrulline (1×10^{-6} to 1×10^{-3} M) were added into the bathing solution; each concentration was applied for 30 min. It produced concentration-dependent decreases in the amplitude of contractile activity of the isolated rat uterus. At the highest dose (1×10^{-3} M), L-citrulline abolished all spontaneous activity. The EC_{50} value of L-citrulline was 64 ± 4.06 μM

(Figure 5.1, n = 6). Thus, the concentration of 64 μM was selected and used throughout the remainder of the study. The inhibitory effects of cumulative doses of L-citrulline on the amplitude, frequency, and AUC are summarized in Table 5.1.

5.4.2 Dose Dependency of L-Arginine

The effects of increasing cumulative concentrations of L-arginine (1×10^{-6} to 1×10^{-3} M) were added into the bathing solution; each concentration was applied for 30 min. As with L-citrulline, it produced concentration-dependent decreases in the amplitude of contractile activity of the isolated rat uterus. The EC_{50} value of L-arginine was 104.12 ± 2.03 μM (Figure 5.1, n = 6). Thus, the concentration of 104 μM was selected and used throughout the remainder of the study. The inhibitory effects of cumulative doses of L-arginine on the amplitude, frequency, and AUC of contractions are summarized in Table 5.2.

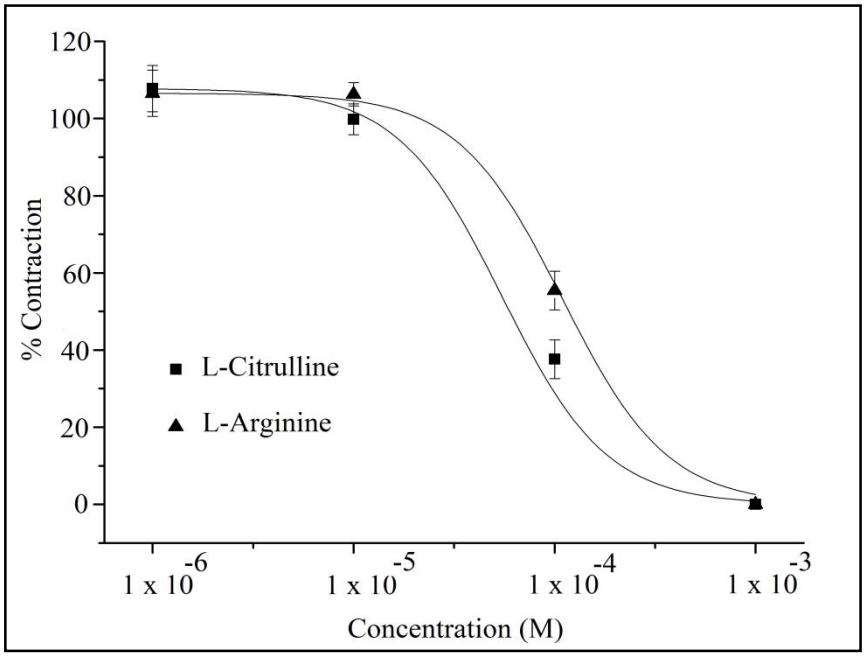

Figure 5.1 Dose dependency of L-citrulline and L-arginine on isolated uterine contraction. Symbol represents means. Vertical lines represent standard errors of the mean (n = 6 for each).

Table 5.1 The effects of L-citrulline at various concentrations on spontaneous contraction.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
L-citrulline (M)				
0 (Control)	100	100	100	6
1×10^{-6}	107.78 ± 6.12	87.49 ± 5.28	91.88 ± 4.46	6
1×10^{-5}	99.83 ± 4.36	79.76 ± 3.86*	78.87 ± 6.23*	6
1×10^{-4}	37.62 ± 5.77*	22.43 ± 3.17*	22.43 ± 5.65*	6
1×10^{-3}	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	6

The *P*-values for amplitude, frequency and AUC of L-citrulline performed are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

Table 5.2 The effects of L-arginine at various concentrations on spontaneous contraction.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
L-arginine (M)				
0 (Control)	100	100	100	6
1×10^{-6}	106.55 ± 6.45	100.25 ± 5.28	103.56 ± 1.98	6
1×10^{-5}	96.31 ± 3.08	72.22 ± 4.36*	73.91 ± 4.17*	6
1×10^{-4}	55.43 ± 5.77*	63.33 ± 6.15*	43.48 ± 3.68*	6
1×10^{-3}	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	6

The *P*-values for amplitude, frequency and AUC of L-arginine performed are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

5.4.3 Effects of L-Citrulline and L-Arginine on Spontaneous Contraction

L-Citrulline

The application of L-citrulline (64 μM) to the rat myometrial preparations produced a significant decrease in the amplitude of force (56.45 ± 5.74% compared with spontaneous contraction, 100%, $P < 0.05$, n = 6). 30 min later, the uterine strip was then washed with Krebs' solution. It was found that the phasic spontaneous contractions of the uterus were reversed. Thus, this finding indicates that the tocolytic effects of L-citrulline on the uterus were reversible. The samples of experimental traces are shown in Figure 5.2A and data summarized in Table 5.3.

L-Arginine

As shown in Figure 5.2B, the application of L-arginine (104 μM) to the rat myometrial preparations produced a significant decrease in the amplitude of force (61.42 ± 1.05% compared with spontaneous contraction, 100%, $P < 0.05$, n = 6). 30 min later, the uterine strip was then washed with Krebs' solution. As with L-citrulline, it was found that the phasic spontaneous contractions of the uterus were reversed. Thus, this finding indicates that the tocolytic effects of L-arginine on the uterus were reversible. The samples of experimental traces are shown in Figure 5.2B and data summarized in Table 5.3.

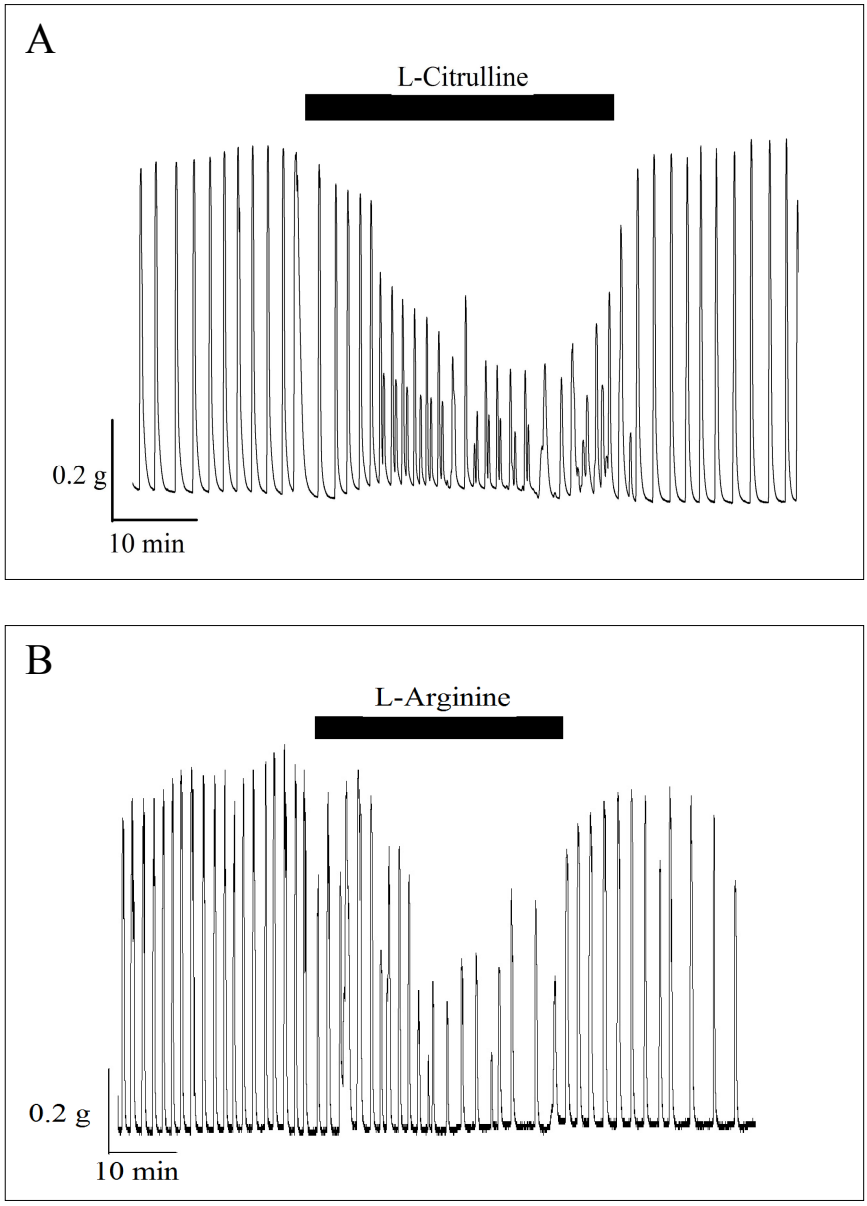

Figure 5.2 The effects of L-citrulline and L-arginine on rat uterine contraction. The applications of 64 µM L-citrulline (A) and 104 µM L-arginine (B) to spontaneous are shown (n = 6 for each).

Table 5.3 The effects of L-citrulline (64 μM) and L-arginine (104 μM) on spontaneous contraction.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
L-citrulline				
Control	100	100	100	6
L-citrulline	56.45 ± 5.74*	117.05± 4.28*	57.95 ± 6.10*	6
Recovery	95.46 ± 6.03	101.22 ± 1.58	93.60 ± 7.01	6
L-arginine				
Control	100	100	100	6
L-arginine	61.42 ± 1.05*	90.05 ± 4.92	60.81 ± 5.68*	6
Recovery	96.48 ± 4.23	92.21 ± 5.38	94.37 ± 3.99	6

The *P*-values for amplitude, frequency and AUC of L-citrulline and L-arginine performed are significantly different from the control ($^{*}P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

5.4.4 Effects of L-Citrulline and L-Arginine on Rat Uterine Contractions in the Presence of L-type Ca^{2+} Channel Activator

L-Citrulline

Uterine contractile force is directly related to the influx of extracellular Ca^{2+} through L-type Ca^{2+} channels (Somlyo and Somlyo, 1998). Thus, the question arose whether the inhibitory effects of L-citrulline on spontaneous contractions were due to the inhibition of L-type Ca^{2+} channels. To do so, the L-type Ca^{2+} channels were activated by using Bay K8644 (1 μM) and the effects of L-citrulline studied. Application of Bay K8644 produced a significant increase in the contraction amplitude (110.09 ± 2.19%) compared with spontaneous contraction (100%, $P < 0.05$, n = 5). Addition of L-citrulline (64 μM) in the continued presence of Bay K8644 produced a marked decrease in the amplitude and the mean integral force to 46.69 ± 4.24% and 49.09 ± 3.53% ($P < 0.05$), respectively, compared with spontaneous contraction (100%). In addition, when Bay K8644 was added after an addition of L-citrulline, it reversed the inhibitory effects of L-citrulline, but the amplitude of contractions did not return to the control level. The amplitude and the mean integral force produced by the combination of L-citrulline and Bay K8644 measured after 10 min application were 83.86 ± 4.38% and 74.00 ± 5.15%, ($P < 0.05$) respectively, compared with spontaneous contraction (100%, n = 8). The samples of experimental traces are shown in Figure 5.3 and data summarized in Table 5.4.

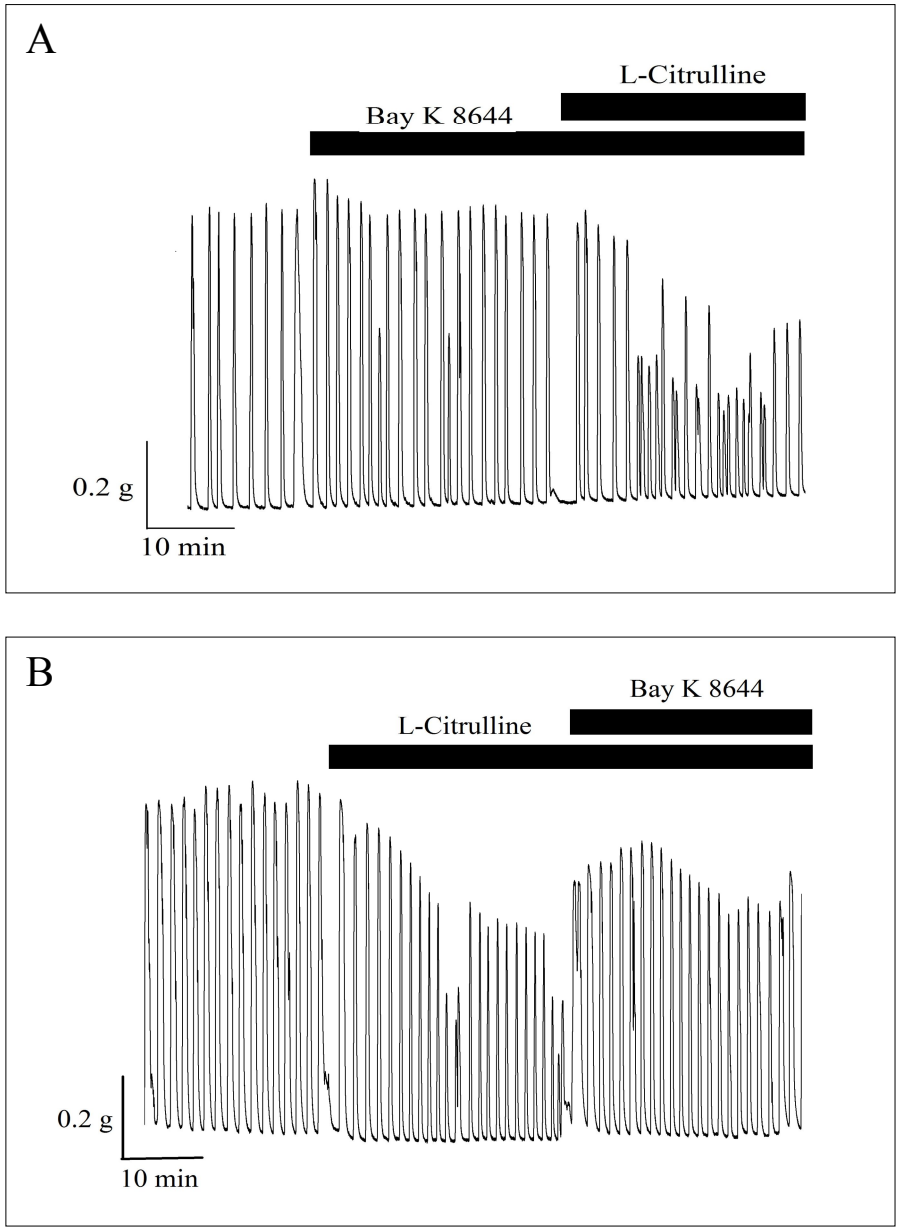

Figure 5.3 The effects of L-citrulline on uterine contraction in the presence of the L-type Ca^{2+} channel activator. Bay K8644 (1 μM) was added before (A, n = 5) and after (B, n = 8) L-citrulline (64 μM).

Table 5.4 The effects of L-citrulline on uterine contraction in the presence of L-type Ca^{2+} channel activator.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
L-citrulline (after)				
Control	100	100	100	5
Bay K8644	110.09 ± 2.19*	140.44± 8.09*	142.44 ± 6.80*	5
Bay K8644 + L-citrulline	46.69 ± 4.24*	120.63 ± 6.13*	49.09 ± 3.53*	5
L-citrulline (before)				
Control	100	100	100	8
L-citrulline	63.25 ± 1.64*	98.09 ± 9.94	60.81 ± 7.58*	8
L-citrulline + Bay K8644	83.86 ± 4.38*	104.75 ± 4.75	74.00 ± 5.15*	8

The *P*-values for amplitude, frequency and AUC of Bay K8644 treated are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

L-Arginine

The effects of L-arginine on uterine contractions in the presence of Bay K8644 were also studied (Figure 5.4). Application of Bay K8644 produced a significant increase in the contraction amplitude (110.10 ± 2.25%, $P < 0.05$) compared with spontaneous contraction (100%, n = 5). Addition of L-arginine in the continued presence of Bay K8644 produced a marked decrease in contraction amplitude and AUC to 51.90 ± 6.27% and 80.91 ± 3.68%, ($P < 0.05$) respectively, compared with spontaneous contraction (100%, n = 5). When Bay K8644 was added after the addition of L-arginine, it did not produce an increase in force. The amplitude and the mean integral force produced by the combination of L-arginine and Bay K8644 measured after 10 min application were 63.15 ± 3.55% and 75.23 ± 3.22%, ($P < 0.05$) respectively, compared with spontaneous contraction (100%, n = 6). The samples of experimental traces are shown in Figure 5.4 and data summarized in Table 5.5.

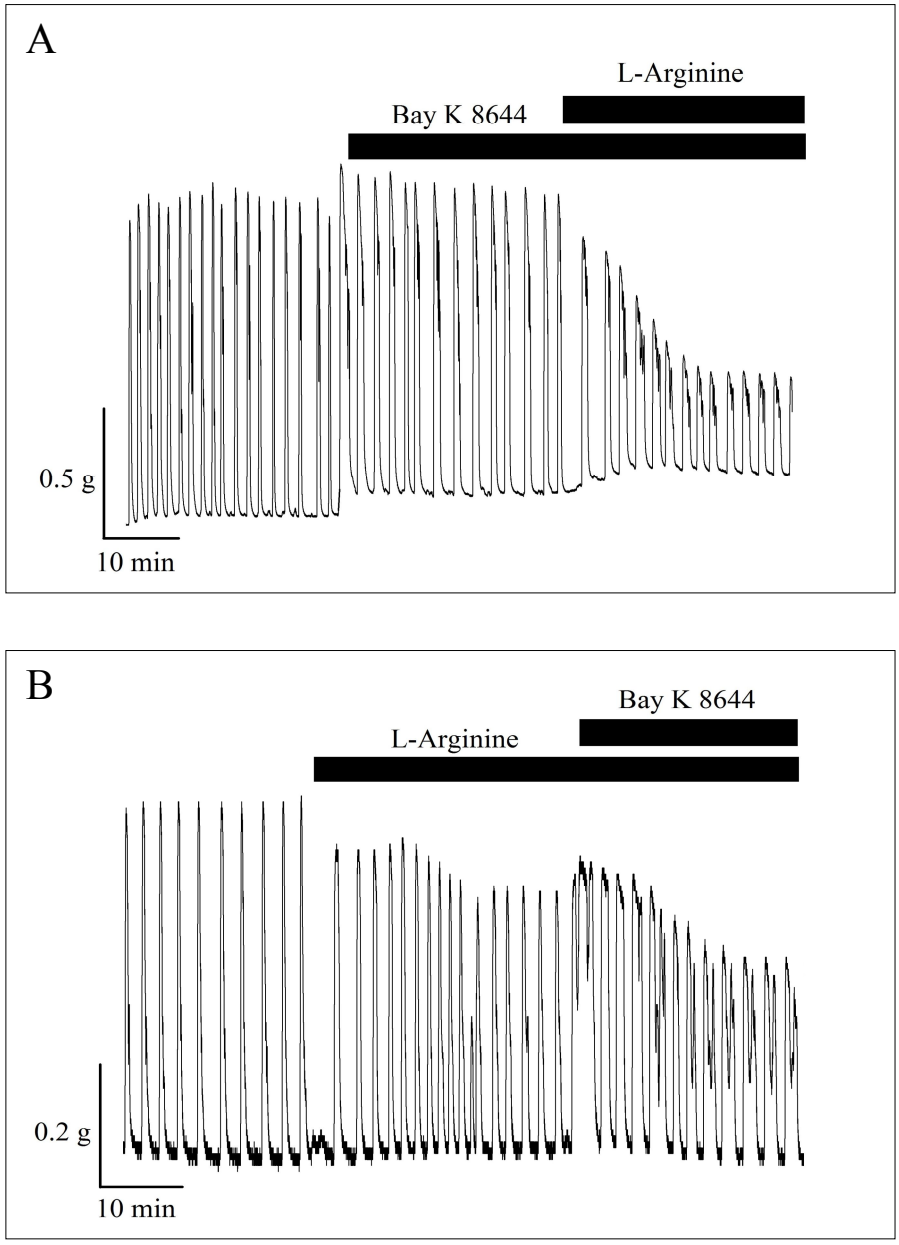

Figure 5.4 The effects of L-arginine on uterine contraction in the presence of the L-type Ca^{2+} channel activator. Bay K8644 (1 μM) was added before (A, n = 5) and after (B, n = 6) L-arginine (104 μM).

Table 5.5 The effects of L-arginine on uterine contraction in the presence of L-type Ca^{2+} channel activator.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
L-arginine (after)				
Control	100	100	100	5
Bay K8644	110.10 ± 2.25	120.12± 2.51*	130.22 ± 6.57*	5
Bay K8644 + L-arginine	51.90 ± 6.27*	100.57 ± 5.29	80.91 ± 3.68*	5
L-arginine (before)				
Control	100	100	100	6
L-arginine	70.46 ± 4.37*	116.25 ± 3.47*	80.48 ± 2.83*	6
L-arginine + Bay K8644	63.15 ± 3.55*	125.13 ± 3.95*	75.23 ± 3.22*	6

The *P*-values for amplitude, frequency and AUC of Bay K8644 treated are significantly different from the control ($^{*}P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

5.4.5 Effects of L-Citrulline and L-Arginine on Rat Uterine Contractions in the Presence of High Ca^{2+}

L-Citrulline

The experiments were performed to verify whether a rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$) could reverse the inhibitory effects of L-citrulline. As can be seen in Figure 5.5A, the application of L-citrulline in the continued presence of high Ca^{2+} decreased the amplitude of contraction to 57.70 ± 5.20% ($P < 0.05$, n = 5) when compared with 100% of spontaneous contraction. When high Ca^{2+} was added after the addition of L-citrulline, it produced an increase in force. The amplitude and the mean integral force produced by the combination of L-citrulline and high Ca^{2+} measured after 10 min application were 77.33 ± 3.30% and 72.59 ± 4.56%, ($P < 0.05$) respectively when compared with spontaneous contraction (100%, n = 5). The samples of experimental traces are shown in Figure 5.5 and data summarized in Table 5.6.

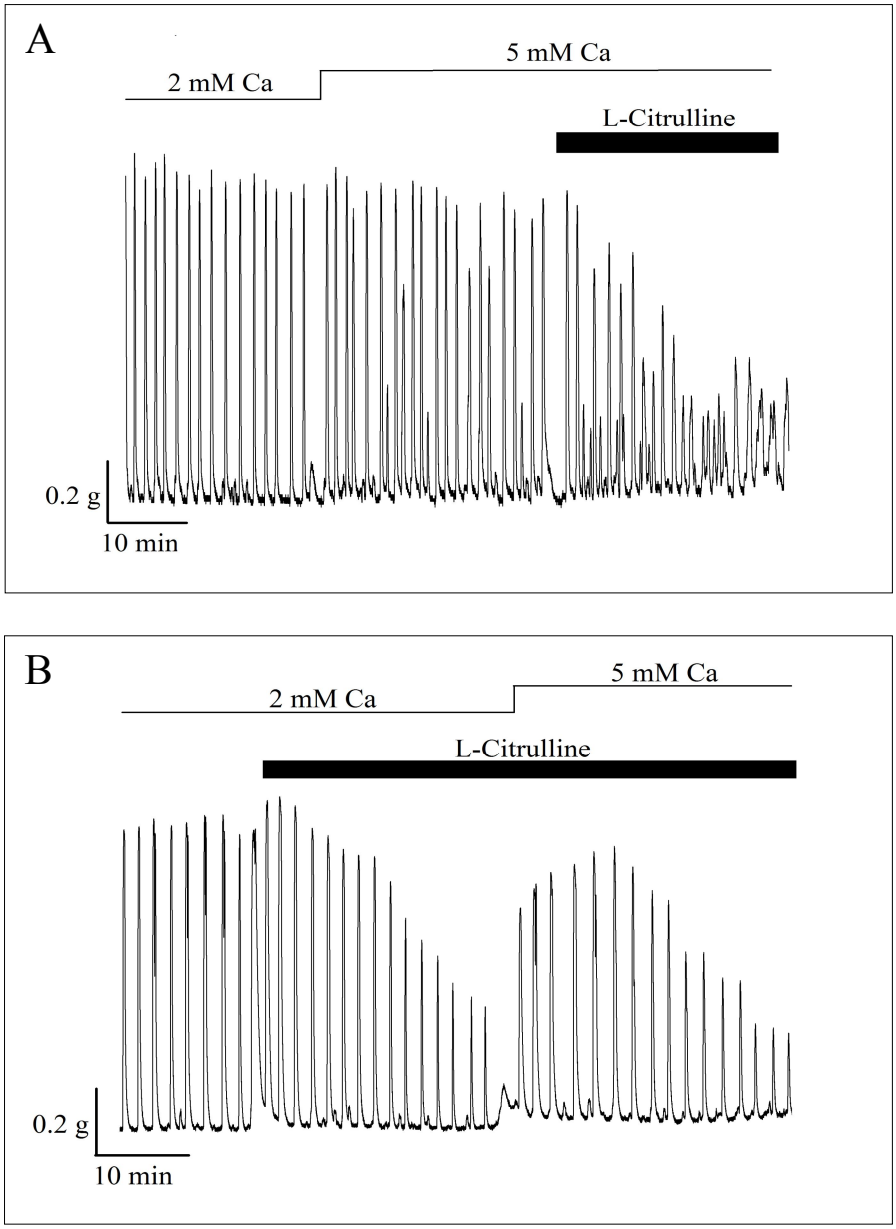

Figure 5.5 The effects of L-citrulline on uterine contraction in the presence of 5 mM $CaCl_2$. 5 mM $CaCl_2$ was added before (A) and after (B) L-citrulline (64 μM) (n = 5 for each).

Table 5.6 The effects of L-citrulline on uterine contraction in the presence of high Ca^{2+}.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
L-citrulline (after)				
Control	100	100	100	5
5 mM $CaCl_2$	114.13 ± 1.44*	116.66± 1.16*	128.38 ± 1.16*	5
5 mM $CaCl_2$ + L-citrulline	57.70 ± 5.20*	114.12 ± 4.33*	65.17 ± 5.63*	5
L-citrulline (before)				
Control	100	100	100	5
L-citrulline	74.13 ± 4.51*	100.25 ± 4.97	69.89 ± 6.12*	5
L-citrulline + 5 mM $CaCl_2$	77.33 ± 3.30*	106.59 ± 4.22	72.59 ± 4.56*	5

The *P*-values for amplitude, frequency and AUC of 5 mM $CaCl_2$ treated are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

L-Arginine

The effects of L-arginine on uterine contraction in the presence of high Ca^{2+} were also studied (Figure 5.6). Addition of L-arginine in the continued presence of high Ca^{2+} produced a decrease in contraction amplitude and the mean integral force to 51.90 ± 6.27% and 80.91 ± 3.68%, ($P < 0.05$) respectively, when compared with spontaneous contraction (100%, n = 5). When high Ca^{2+} was added after the addition of L-arginine, it did not produce an increase in force. The amplitude and the mean integral force produced by the combination of L-arginine and Bay K8644 measured after 10 min application were 69.00 ± 1.05% and 85.26 ± 2.93%, ($P < 0.05$) respectively when compared with spontaneous contraction (100%, n = 6). The samples of experimental traces are shown in Figure 5.6 and data summarized in Table 5.7.

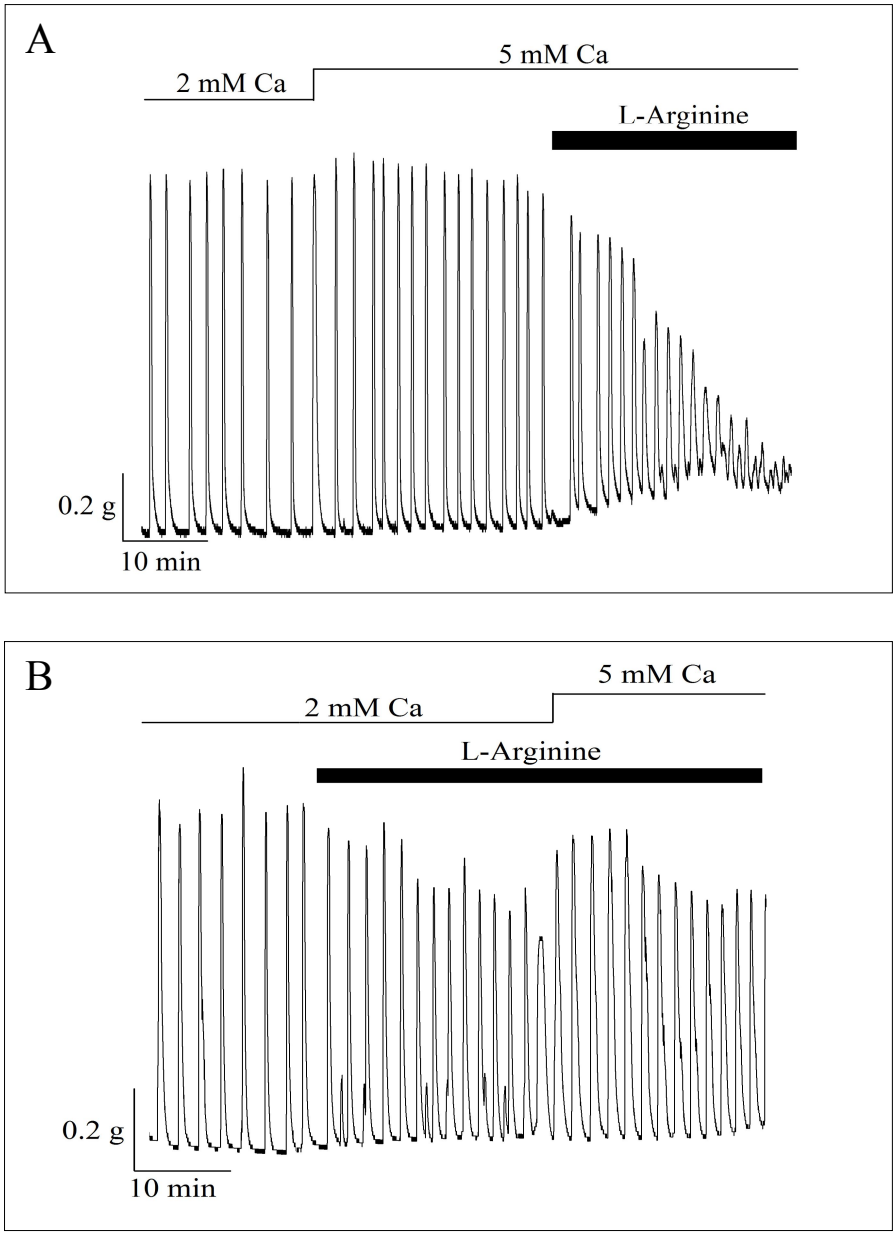

Figure 5.6 The effects of L-arginine on uterine contraction in the presence of 5 mM $CaCl_2$. 5 mM $CaCl_2$ was added before (A) and after (B) L-arginine (104 μM) (n = 5 for each).

Table 5.7 The effects of L-arginine on uterine contraction in the presence of high Ca^{2+}.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
L-arginine (after)				
Control	100	100	100	5
5 mM $CaCl_2$	111.96 ± 3.29*	125.36± 3.65*	115.70 ± 3.96*	5
5 mM $CaCl_2$ + L-arginine	69.00 ± 1.05*	125.10 ± 3.06*	75.23 ± 3.06*	5
L-arginine (before)				
Control	100	100	100	6
L-arginine	76.86 ± 6.55*	120.64 ± 6.46*	79.28 ± 2.60*	6
L-arginine + 5 mM $CaCl_2$	83.17 ± 3.25*	125.25 ± 3.25*	85.26 ± 2.93*	6

The *P*-values for amplitude, frequency and AUC of 5 mM $CaCl_2$ treated are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

5.4.6 Effects of L-Citrulline and L-Arginine on $PGF_{2\alpha}$-Induced Uterine Contraction

L-Citrulline

$PGF_{2\alpha}$ causes uterine contraction by acting on prostaglandin FP receptor, which results in the release of Ca^{2+} from the intracellular store (SR) (Phillippe and Chien, 1998). As can be seen in Figure 5.7A, the application of $PGF_{2\alpha}$ (1 μM) significantly increased the amplitude, the frequency and the mean integral force of the contractions. Application of L-citrulline (64 μM) to the myometrium in the continued presence of $PGF_{2\alpha}$ exerted significant inhibitory effects on both amplitude and the mean integral force (n = 5). The samples of experimental traces are shown in Figure 5.7A and data summarized in Table 5.8.

L-Arginine

As can be seen in Figure 5.7B, 1 μM $PGF_{2\alpha}$ was significantly increased the amplitude, the frequency and the mean integral force of the contractions. Application of L-arginine (104 μM) to the myometrium in the continued presence of $PGF_{2\alpha}$ exerted significant inhibitory effects on both amplitude and the mean integral force (n = 5). The samples of experimental traces are shown in Figure 5.7B and data summarized in Table 5.8.

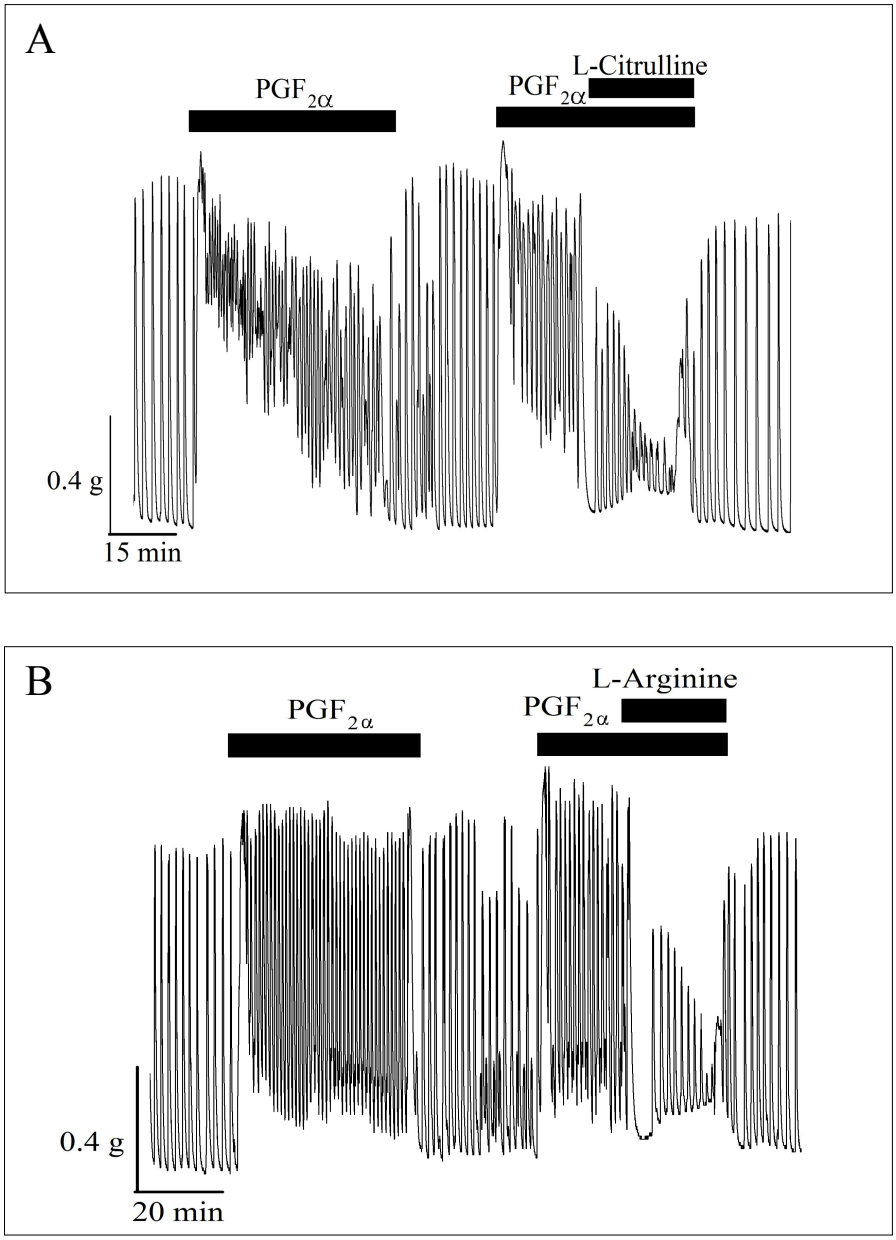

Figure 5.7 The effects of L-citrulline and L-arginine on $PGF_{2\alpha}$-induced uterine contraction. The effects of 64 µM L-citrulline (A) and 104 µM L-arginine (B) on uterine contraction-induced by 1 µM $PGF_{2\alpha}$ are shown (n = 5 for each).

Table 5.8 The effects of L-citrulline and L-arginine on $PGF_{2\alpha}$-induced uterine contraction.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
L-citrulline				
Control	100	100	100	5
$PGF_{2\alpha}$	125.39 ± 1.15*	130.54 ± 1.17*	134.76 ± 5.40*	5
$PGF_{2\alpha}$ + L-citrulline	56.92 ± 5.44*	116.23 ± 4.29*	76.78 ± 3.79*	5
L-arginine				
Control	100	100	100	5
$PGF_{2\alpha}$	128.20 ± 1.23*	128.57 ± 3.20*	143.76 ± 8.14*	5
$PGF_{2\alpha}$ + L-arginine	61.48 ± 4.74*	114.28 ± 3.48*	60.43 ± 7.05*	5

The *P*-values for amplitude, frequency and AUC of 1 μM $PGF_{2\alpha}$ treated are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

5.4.7 Effects of L-Citrulline and L-Arginine on Oxytocin-Induced Uterine Contraction

L-Citrulline

Oxytocin is a nonapeptide hormone, which is known to mediate both directly and indirectly to stimulate uterine smooth muscle contraction (Phillippe and Chien, 1998). Oxytocin causes uterine contraction by acting on oxytocin receptor, which results in the Ca^{2+} influx through L-type Ca^{2+} channel and the release of Ca^{2+} from the SR (Phillippe and Chien, 1998). As can be seen in Figure 5.8A, 10 nM oxytocin significantly increased the amplitude, the frequency and the mean integral force of the contractions. Application of L-citrulline (64 μM) to the myometrium in the continued presence of 10 nM oxytocin produced a significant inhibition on both the amplitude and the mean integral force (n = 5). The samples of experimental traces are shown in Figure 5.8A and data summarized in Table 5.9.

L-Arginine

As can be seen in Figure 5.8B, 10 nM oxytocin significantly increased the amplitude, the frequency and the mean integral force of the contractions. Application of L-arginine (104 μM) to the myometrium in the continued presence of oxytocin caused a significant inhibition on both the amplitude and the mean integral force (n = 5). The samples of experimental traces are shown in Figure 5.8B and data summarized in Table 5.9.

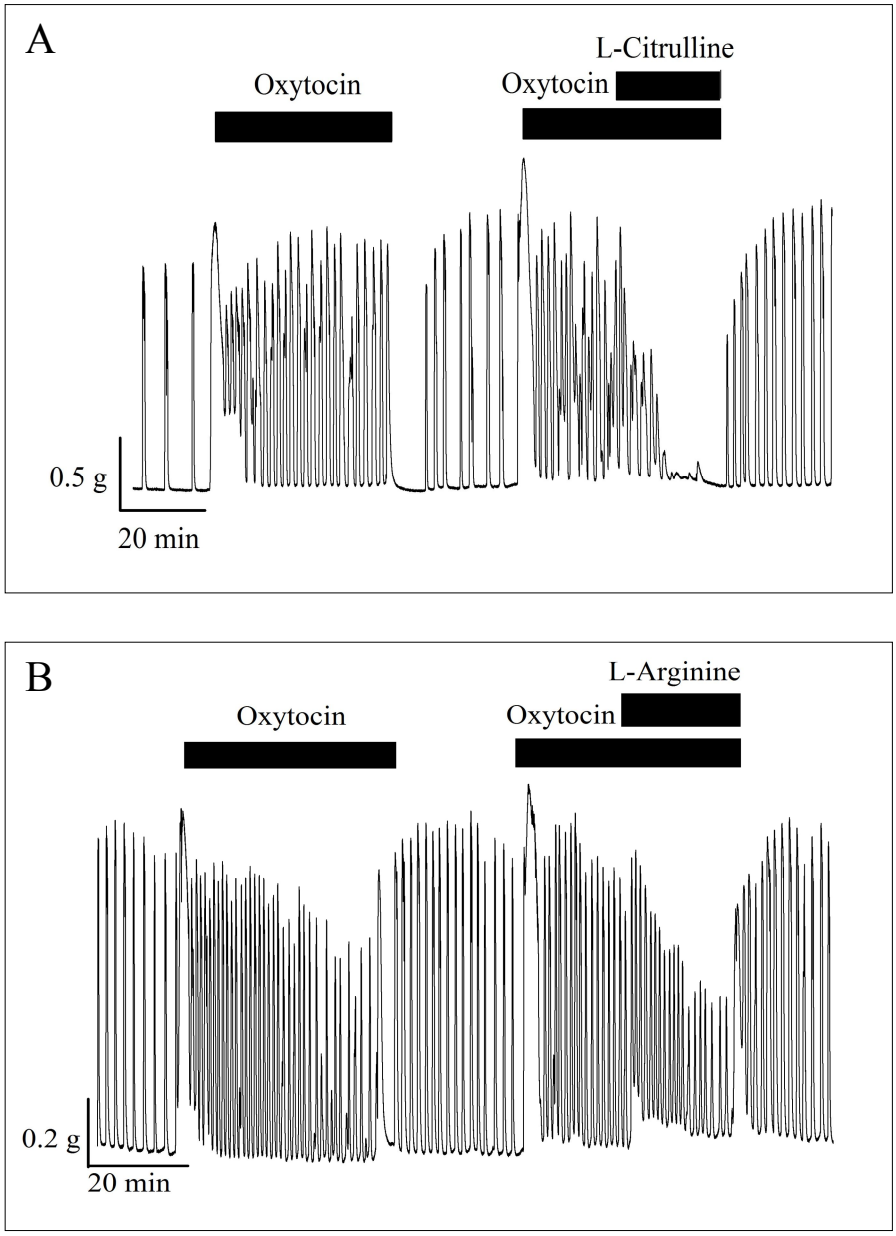

Figure 5.8 The effects of L-citrulline and L-arginine on oxytocin-induced uterine contraction. The effects of 64 µM L-citrulline (A) and 104 µM L-arginine (B) on uterine contraction-induced by 10 nM oxytocin are shown (n = 5 for each).

Table 5.9 The effects of L-citrulline and L-arginine on oxytocin-induced uterine contraction.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
L-citrulline				
Control	100	100	100	5
Oxytocin	128.63 ± 4.09*	150.05± 6.72*	158.05 ± 2.80*	5
Oxytocin + L-citrulline	65.51 ± 3.64*	120.82 ± 6.09*	78.46 ± 1.56*	5
L-arginine				
Control	100	100	100	5
Oxytocin	125.35 ± 5.03*	150.42 ± 4.76*	148.26± 8.36*	5
Oxytocin + L-arginine	68.46 ± 5.63*	116.12 ± 2.80*	65.59 ± 4.34*	5

The *P*-values for amplitude, frequency and AUC of 10 nM oxytocin treated are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

5.4.8 Effects of L-Citrulline and L-Arginine on KCl-Induced Uterine Contraction

L-Citrulline

In most tissues, the KCl-induced contraction is highly sensitive to the inhibitors of Ca^{2+} channels and abolishes in 0-Ca solution. Thus, isoosmotic KCl solution-induced contraction is mainly mediated by Ca^{2+} entry through voltage-dependent Ca^{2+} channels (Ausina, Savineau, Pinto, Martin and Candenas, 1996). Figure 5.9A shows a representative tracing of the KCl (40 mM)-induced uterine contraction. The addition of KCl elicited an initial rapid phasic contraction followed by a sustained tonic contraction. Upon return to control solution, spontaneous phasic contractions reappeared after a delay of approximately 5 min. 30 min later, the solution was changed to KCl. This resulted initially in a rise of tension to approximately the same level as seen with the first treatment of KCl. L-citrulline (64 μM) was applied to the myometrium during the sustained tonic contraction. It produced a significant inhibition of the contraction (n = 7). This value was 62.66 ± 4.66% ($P < 0.05$) compared to the KCl control (100%). The samples of experimental traces are shown in Figure 5.9A.

L-Arginine

As can be seen in Figure 5.9B, isoosmotic KCl (40 mM) solution significantly increased the contractions. Application of L-arginine (104 μM) to the myometrium in the continued presence of KCl caused a significant inhibition on force (n = 7). This value was 66.38 ± 2.23% ($P < 0.05$) compared to the KCl control (100%). The samples of experimental traces are shown in Figure 5.9B.

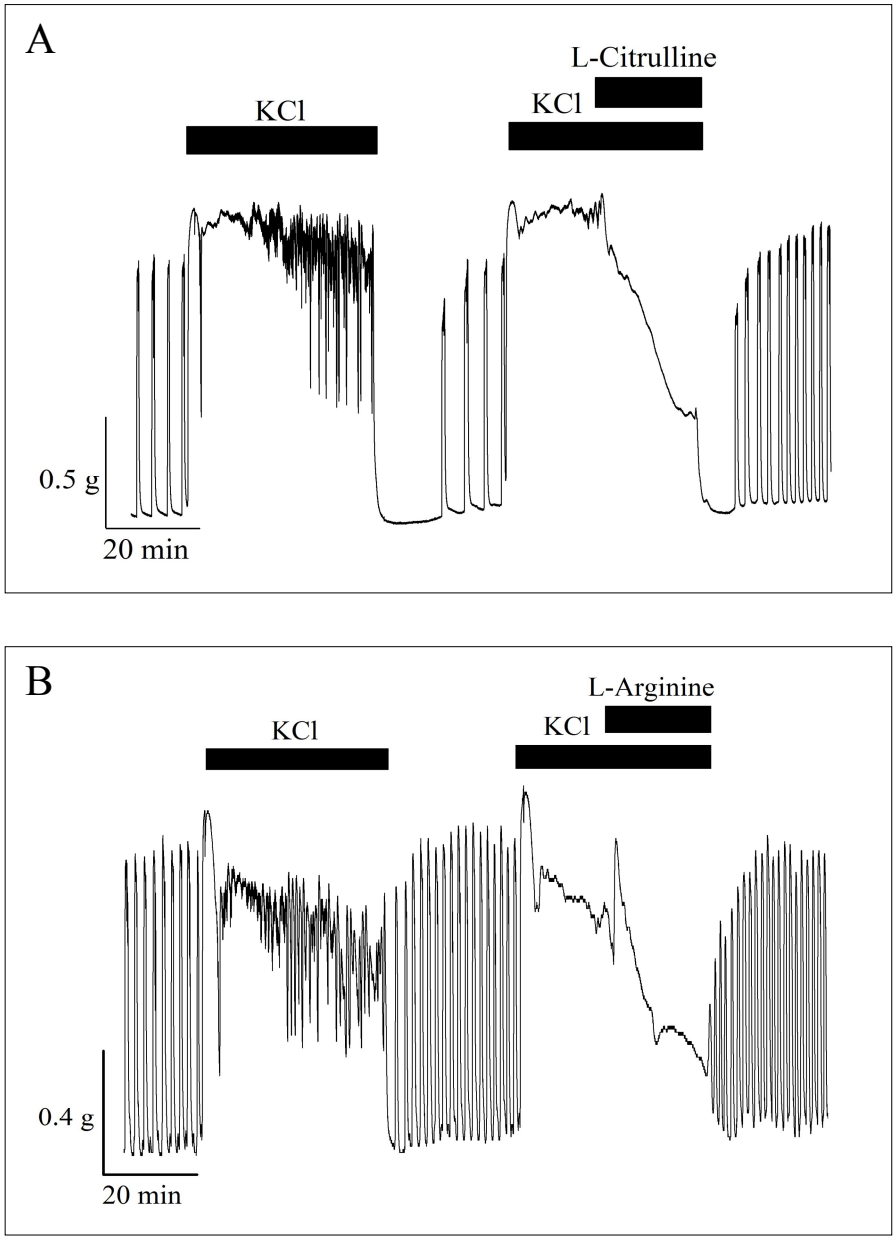

Figure 5.9 The effects of L-citrulline and L-arginine on KCl-induced uterine contraction. The effects of 64 µM L-citrulline (A) and 104 µM L-arginine (B) on uterine contraction-induced by KCl (40 mM) are shown (n = 7 for each).

5.4.9 Effects of L-Citrulline and L-Arginine on $PGF_{2\alpha}$-Induced Uterine Contraction in the Absence of External Ca^{2+}

L-Citrulline

The contraction of uterine smooth muscles depends on the presence of external Ca^{2+}. It has been found that Ca^{2+}-removal rapidly abolished the phasic contractions (Matthew, Kupittayanant, Burdyga and Wray, 2004; Kupittayanant et al., 2002). Interestingly, some agonists can induce contraction in the absence of external Ca^{2+}. These contractions are generally interpreted as being due to Ca^{2+} released from the SR (Kupittayanant et al., 2002; Wray et al., 2003). Thus, it was of interest to verify whether L-citrulline could alter this contraction. $PGF_{2\alpha}$ (1 μM) was added in the absence of external Ca^{2+} entry. This protocol was performed to ensure that the only source of Ca^{2+} was from the SR (Matthew et al., 2004; Kupittayanant et al., 2002). As can be seen in Figure 5.10A, phasic contractions abolished upon changing to 0-Ca solution. In the continued presence of 0-Ca solution, 1 μM $PGF_{2\alpha}$ produced a small tonic force as long as this agonist was present. Upon return to control solution, spontaneous phasic contractions reappeared. 30 min later, the solution was changed to one containing 0-Ca and L-citrulline. Force abolished rapidly and $PGF_{2\alpha}$ added. It was found that very little if any force was produced (Figure 5.10A, n = 6). This value was 74.33 ± 3.75% ($P < 0.05$) compared to $PGF_{2\alpha}$-induced contraction in the absence of external Ca^{2+} alone (100%, n = 6). The samples of experimental traces are shown in Figure 5.10A.

L-Arginine

The effects of L-arginine on $PGF_{2\alpha}$-induced uterine contraction in the absence of external Ca^{2+} were also examined. The effects were similarly to the effects of L-citrulline. $PGF_{2\alpha}$ produced a small tonic force. L-arginine reduced this contraction to 78.72 ± 2.82% ($P < 0.05$) compared to $PGF_{2\alpha}$-induced contraction in the absence of external Ca^{2+} alone (100%, n = 6). The samples of experimental traces are shown in Figure 5.10B.

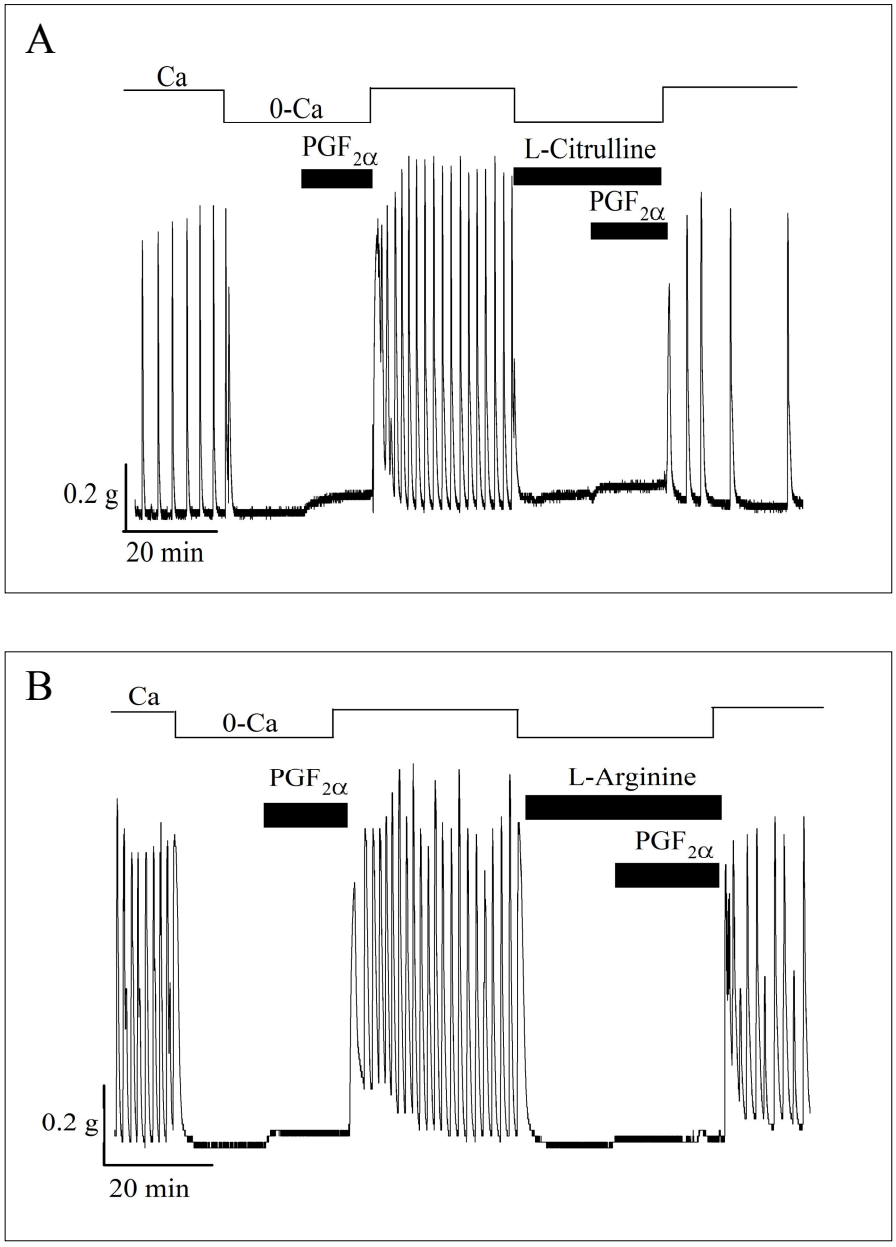

Figure 5.10 The effects of L-citrulline and L-arginine on $PGF_{2\alpha}$-induced uterine contraction in the absence of external Ca^{2+}. The effects of 64 μM L-citrulline (A) and 104 μM L-arginine (B) on uterine contraction-induced by 1 μM $PGF_{2\alpha}$ in 0-Ca solution are shown (n = 6 for each).

5.4.10 Effects of L-Citrulline and L-Arginine on Oxytocin-Induced Uterine Contraction in the Absence of External Ca^{2+}

L-Citrulline

The same protocol was used as described above for $PGF_{2\alpha}$. In 0-Ca solution, 10 nM oxytocin generated a small tonic force, indicating that oxytocin can release Ca^{2+} from the SR. Application of 64 μM L-citrulline caused a significant inhibition of the contraction induced by oxytocin in the absence of external Ca^{2+} (Figure 5.11A). This value was 70.25 ± 9.03% ($P < 0.05$) compared to oxytocin-induced contraction in the absence of external Ca^{2+} alone (100%, n = 6). The samples of experimental traces are shown in Figure 5.11A.

L-Arginine

Application of L-arginine (104 μM) to uterine contraction-induced by oxytocin in the absence of external Ca^{2+} produced a marked significant inhibition of the contraction (Figure 5.11B). This value was 77.36 ± 4.07% ($P < 0.05$) compared to oxytocin-induced contraction in the absence of external Ca^{2+} alone (100%, n = 6). The samples of experimental traces are shown in Figure 5.11B.

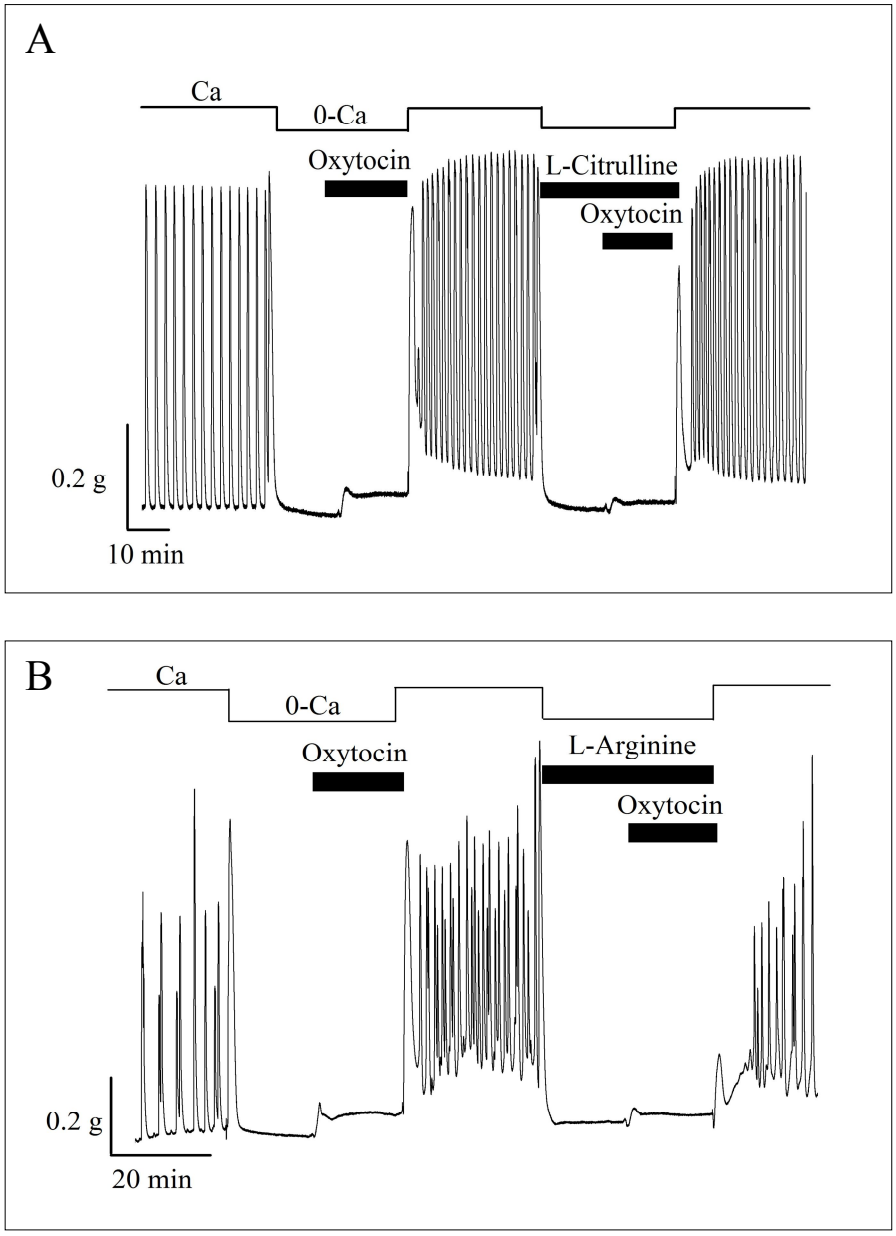

Figure 5.11 The effects of L-citrulline and L-arginine on oxytocin-induced uterine contraction in the absence of external Ca^{2+}. The effects of 64 μM L-citrulline (A) and 104 μM L-arginine (B) on uterine contraction-induced by 10 nM oxytocin in 0-Ca solution are shown (n = 6 for each).

5.4.11 Effects of L-Citrulline and L-Arginine on Oxytocin-Induced Uterine Contraction in the Presence of KCl

L-Citrulline

It has been reported that, when Ca^{2+} was high or maintained, smooth muscle contraction may be modulated by Ca^{2+}-independent pathway. For example, application of oxytocin in the presence of KCl produced a tonic component of force, indicating release of Ca^{2+} from the SR. In addition, it was suggested that the potentiation of force by oxytocin may due to the modulation of myosin light chain phosphatase (MLCP) activity through rho-associated kinase (ROK) pathway (Kupittayanant, Burdyga and Wray, 2001; Somlyo and Somlyo, 1998). ROK stimulated by oxytocin produced a marked increase in force without a change in $[Ca^{2+}]_i$ (Kupittayanant et al., 2001). Thus, it was of interest to verify whether L-citrulline may alter this kind of contraction; oxytocin-induced uterine contraction in the presence of KCl. As can be seen in Figure 5.12A, application of oxytocin (10 nM) in the continued presence of KCl produced a small tonic contraction, suggesting the release of Ca^{2+} from the SR. Upon return to control solution, uterine tension quickly returned to baseline and spontaneous phasic contractions reappeared. 30 min later, the solution was then changed to KCl and oxytocin added. In addition, L-citrulline (64 μM) was applied to the myometrium during the sustained tonic contraction. It produced a marked decrease in force. Thus, after 10 min, force had fallen to 60.66 ± 4.66% of control force development ($P < 0.05$, 100% force was the level of maintained force that was produced during the application of 10 nM oxytocin in the

presence of 40 mM KCl, n = 6). The samples of experimental traces are shown in Figure 5.12A.

L-Arginine

The same protocol was used as described above for L-citrulline. Application of L-arginine (104 μM) was able to decrease force upon oxytocin application to the uterus in the presence of KCl. After 10 min, force had fallen to 65.99 ± 7.46% of control force development ($P < 0.05$, 100% force was the level of maintained force that was produced during the application of 10 nM oxytocin in the presence of 40 mM KCl solution, n = 6). The samples of experimental traces are shown in Figure 5.12B.

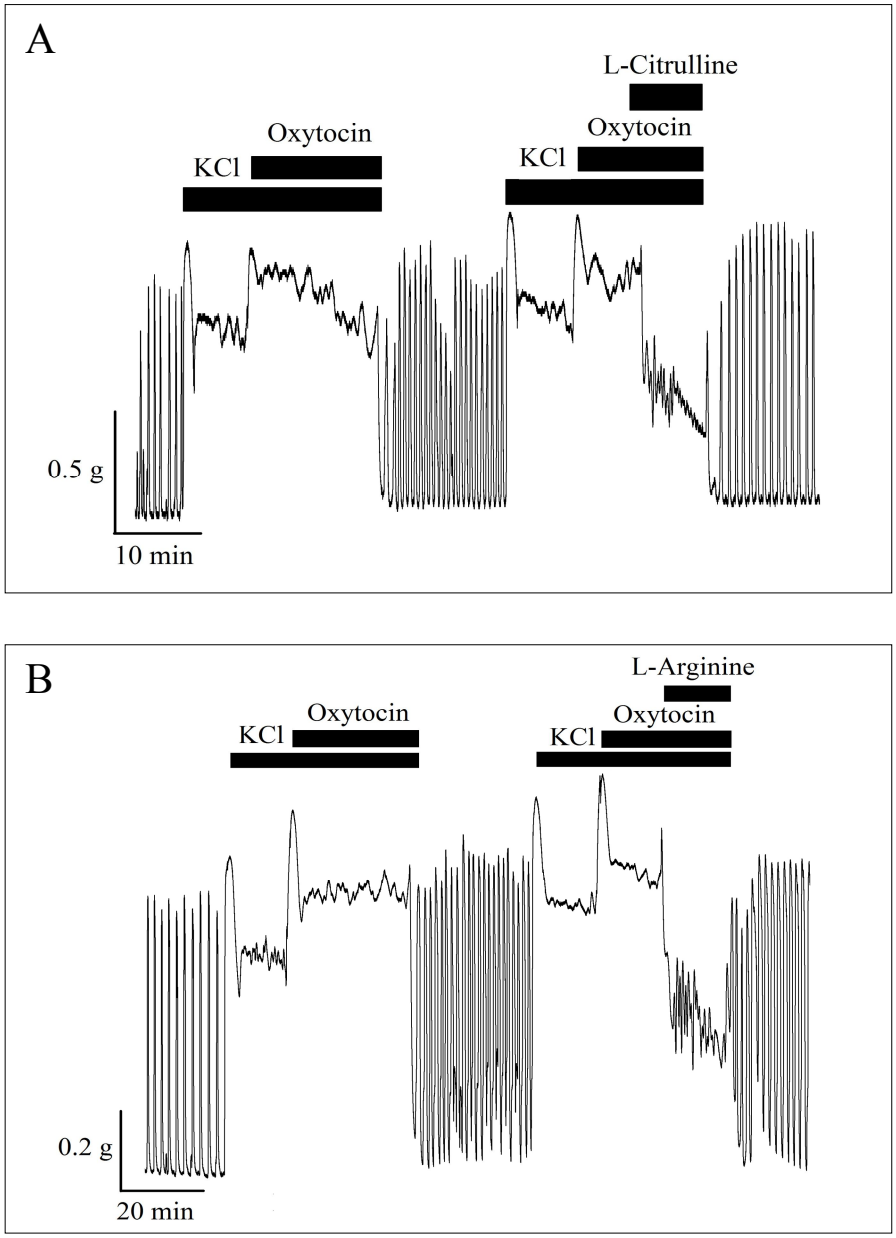

Figure 5.12 The effects of L-citrulline and L-arginine on oxytocin-induced uterine contraction in the presence of KCl. The effects of 64 μM L-citrulline (A) and 104 μM L-arginine (B) on oxytocin-induced uterine contraction in the presence of 40 mM KCl solution are shown (n = 6 for each).

5.5 Discussion

It has been reported that watermelon is rich in L-citrulline and L-arginine. Thus, the aim of this chapter was to verify whether the inhibitory effects of watermelon extracts on uterine contraction as shown in the previous chapters were due to L-citrulline and L-arginine. This study demonstrated the effects of L-citrulline and L-arginine on uterine contraction and revealed that both L-citrulline and L-arginine effectively depressed spontaneous contractions. In addition, L-citrulline and L-arginine caused inhibition of both the amplitude and the mean integral force of the contractions induced by $PGF_{2\alpha}$, oxytocin and KCl. In the absence of external Ca^{2+}, L-citrulline and L-arginine produced partially inhibition of contraction induced by $PGF_{2\alpha}$ and oxytocin. Moreover, L-citrulline and L-arginine altered the tonic component of force produced by the application of oxytocin to the uterus in the continued presence of KCl. These findings suggest that the tocolytic effects of L-citrulline and L-arginine on myometrial contractions were dependent upon Ca^{2+}-dependent and Ca^{2+}-independent regulation of smooth muscle contraction that were similarly to those of watermelon extracts.

Myometrial contractions are phasic in nature (Kupittayanant et al., 2001). The mechanism that causes phasic activity is alteration of the potential across the myometrial cell membrane (Wray et al., 2003). It is indicated that the level of $[Ca^{2+}]_i$ is important for the modulation of force (Kupittayanant et al., 2002). The main sources of Ca^{2+} are 1) the release of the SR and 2) the influx of extracellular Ca^{2+} through the L-type Ca^{2+} channels. The increased in $[Ca^{2+}]_i$ results in calmodulin activation of myosin light chain kinase (MLCK). This kinase phosphorylates the

regulatory light chains of myosin, leading to the activation of myosin MgATPase and force development (Kupittayanant et al., 2002; Phillippe and Edward, 1998; Somlyo and Somlyo, 1998). The application of L-citrulline and L-arginine to the uterine strips produced a dose dependent inhibition of spontaneous phasic contractions. These findings suggest that L-citrulline and L-arginine inhibit the contraction mainly by interrupting the influx of Ca^{2+}.

The activation of L-type Ca^{2+} channels by some agonists or high Ca^{2+} leads to the influx of Ca^{2+} into the cell and contractions (Buddhakala et al., 2008; Kupittayanant et al., 2008; Wray et al., 2003). The addition of L-citrulline or L-arginine to the myometrium in the continued presence of 5 mM $CaCl_2$ and Bay K8644 caused a marked decrease in uterine force. Moreover, adding of 5 mM $CaCl_2$ or Bay K8644 to the uterus in the presence of L-citrulline or L-arginine partially reversed the inhibitory effects of these two amino acids. Thus, these findings suggest that the tocolytic effects of L-citrulline and L-arginine may be associated with, at least in part, the inhibition of L-type Ca^{2+} channels.

It is well established that KCl induces a rapid rise in $[Ca^{2+}]_i$ through depolarization of the cell membrane, leading to the opening of L-type Ca^{2+} channels and muscle contraction (Ausina et al., 1996; Kupittayanant et al., 2001). Some substances that inhibit the KCl-induced contractions mediate through blocking voltage-dependent Ca^{2+} channels (Gharib Naseri and Yahyavi, 2007). The addition of L-citrulline and L-arginine to the uterus was able to partially inhibit contractility in the presence of KCl, supporting the hypothesis that these two amino acids possess a Ca^{2+} entry blocking activity.

Oxytocin and $PGF_{2\alpha}$ produce uterine contractions by acting on oxytocin and prostaglandin FP receptors, respectively (Phillippe and Edward, 1998; Soloff, 1990). These receptors are connected with $G\alpha_{q/11}$ subunit to membrane phospholypase Cβ (PLCβ). It is well established that the catalyzed PLCβ hydrolyses the membrane phosphatidylinositol-4,5-bisphosphate, resulting in the creation of two parallel signaling pathway: IP_3 and DAG (Phillippe and Edward, 1998; Soloff, 1990). IP_3 binds to its receptor on the SR membrane and causes release of Ca^{2+} into the cytoplasm (Phillippe and Edward, 1998; Soloff, 1990). In addition, it has been reported that oxytocin itself elevates $[Ca^{2+}]_i$ by activating the L-type Ca^{2+} channels and chloride (Cl_{Ca}) channels (Arnaudeau, Lepretre and Mironneau, 1994; Wray et al., 2003). The activation of Cl_{Ca} channels will result in depolarization, as Cl^- leaves the cell. Thus, these channels may play a crucial role in regulating membrane excitability and increasing the Ca^{2+} entry through voltage-dependent Ca^{2+} channels (Arnaudeau et al., 1994; Wray et al., 2003).

The application of $PGF_{2\alpha}$ and oxytocin to spontaneous phasic contractions induced a rapid rise in the amplitude, the frequency, and the mean integral force of the contractions. The application of L-citrulline and L-arginine to $PGF_{2\alpha}$-induced or oxytocin-induced uterine contraction caused a marked inhibition of the force of contraction. These findings indicate that the spasmolytic activity of L-citrulline and L-arginine may be due to the interruption of $G\alpha_{q/11}$-PLCβ-IP_3 pathway and the inhibition of Ca^{2+} entry.

It has been reported that L-citrulline and L-arginine mediated relaxation in smooth muscle through NO-cGMP pathway modulation (Norman, 1996). NO is synthesized by the catalytic action of a family of NO synthase (NOS) that convert the

substrate, L-arginine, to NO and L-citrulline (Flam et al., 2001; Lincoln et al., 1995). Some investigators indicated that L-citrulline can convert to L-arginine in all cell types through specific enzymes, argininosuccinate synthase and argininosuccinate lyase (Romero et al., 2006). NO induces changes in target proteins through the activation of cGMP. The mechanisms underlying of NO and cGMP could affects $[Ca^{2+}]_i$ in four different ways: 1) by reducing Ca^{2+}; 2) by increasing Ca^{2+} efflux; 3) by enhancing Ca^{2+} sequestration; and 4) by decreasing Ca^{2+} mobilization (Lincoln et al., 1995; McDonald and Murad, 1995; Norman, 1996). Based on these scientific evidences, it is possible to speculate that NO-cGMP pathway modulation may also be involved in the inhibitory effects of L-citrulline and L-arginine on rat uterine contraction in this present study.

In the presence of 0-Ca solution, uterine force was rapidly abolished. The application of $PGF_{2\alpha}$ or oxytocin to the myometrium in the absence of external Ca^{2+} produced a small tonic force, indicating the ability of $PGF_{2\alpha}$ or oxytocin to release Ca^{2+} from the SR and to produce uterine force in these conditions (Matthew et al., 2004; Kupittayanant et al., 2002). L-citrulline and L-arginine altered the contraction triggered by $PGF_{2\alpha}$ or oxytocin in 0-Ca solution, because pre-incubation with L-citrulline or L-arginine significantly inhibited $PGF_{2\alpha}$-or oxytocin-induced contraction approximately 70%. These findings suggest that L-citrulline and L-arginine provoked a reduction on the SR release by interfering in $G\alpha_{q/11}$-PLCβ-IP_3 pathway. Some investigators indicated that the cyclic nucleotide analogue 8-bromo cGMP also inhibited contraction through the suppression of phosphatidylinositol hydrolysis (Rapoport, 1986). It is thought that inhibition of phosphatidylinositol breakdown mediated by cGMP may be due to the activation of cGMP-dependent

protein kinase (PKG) (Lincoln et al., 1995; Rapoport, 1986). It was indicated that PKG catalyzes the phosphorylation of the IP_3 receptor at Serine 1755, resulting in inhibition of the release of Ca^{2+} from the SR (Komalavilas and Lincoln, 1994; Lincoln et al., 1995). The present finding demonstrated that L-citrulline and L-arginine decreased the contractions-induced by $PGF_{2\alpha}$ and oxytocin in a 0-Ca solution, which suggested that these amino acids could inhibit the IP_3-dependent Ca^{2+} release through the mechanisms described above.

A major modulation of uterine contraction is the level of myosin light chain phosphorylation that is modulated by MLCK and MLCP. An increased tension and phosphorylation can occur at constant Ca^{2+} and is usually known as Ca^{2+} sensitization (Somlyo and Somlyo, 1998). This prevalence occurs frequently after stimulation by many agonists, including oxytocin. The mechanism underlying of this effect might be due to the inhibition of MLCP by the ROK pathway (Somlyo and Somlyo, 1998). It has been shown that Rho kinase inhibitors, such as Y-27632, block the agonist-induced Ca^{2+} sensitization in human myometrium (Kupittayanant et al., 2001). Application of Y-27632 to the phasic spontaneous contraction had very little effect on force. However, when the uterine strips were produced a tonic contraction, Y-27632 significantly decreased in force without a change in $[Ca^{2+}]_i$. This study suggested that the Rho pathway is more important in promoting force development during tonic rather than phasic contractions (Kupittayanant et al., 2001). In this regard, when the myometrium strips were pre-incubated with KCl, then added a physiological dose of oxytocin, it potentiated a tonic component of force. Under these conditions it is expected that L-type Ca^{2+} channels will be maximally open by KCl, and oxytocin will stimulate receptor and store operated Ca^{2+} entry pathways and SR Ca^{2+} release. In

addition, it is thought that the inhibition of MLCP mediated by Rho kinase may also be served as the mechanisms of oxytocin to induce increases in myometrial contractility under these circumstances (Shmygol, Gullam, Blanks and Thornton, 2006). The application of L-citrulline or L-arginine to oxytocin-induced contraction in the presence of KCl produced a marked decrease in force. These findings indicate that the tocolytic effects of L-citrulline and L-arginine might be associated with the inhibition of the ROK pathway.

To the best of our knowledge, this present study firstly showed the tocolytic effects of L-citrulline on rat uterine contraction. It was observed that the effects of L-citrulline were slightly larger than the effects of L-arginine. This assessment may be associated with the saturation of the substrate, L-arginine, for NOS (Flam et al., 2001). It was exhibited that caveolae may be the principle source of L-arginine available to NOS (Flam et al., 2001). In addition, the L-citrulline to L-arginine recycling pathway is localized to caveolae and may serve as the principle source of available L-arginine (Flam et al., 2001). Thus, a key observation was that the inhibitory effects of L-citrulline, in part or entirely, shows the L-arginine-like pharmacological effects as previously described by several investigators (Cormio et al., 2011; Hoffmann et al., 2003; Ochiai et al., 2010; Ranghavan and Dikshit, 2001; Romero et al., 2006).

Based on the results of present study, L-citrulline and L-arginine produced a significant suppressive effect on oxytocin-induced contraction, suggesting that L-citrulline and L-arginine may be effective in the prevention of preterm labor (Shmygol et al., 2006; Soloff, 1990). In addition, both L-citrulline and L-arginine

exhibited the inhibitory effects on $PGF_{2\alpha}$-induced contraction, suggesting that they may also be effective in the treatment of primary dysmenorrhea (Harel, 2006).

In conclusion, this study investigated the tocolytic effects of L-citrulline and L-arginine on isolated rat uterus. The results revealed that L-citrulline and L-arginine had a dose-dependent effect on the isolated rat uterus. L-citrulline and L-arginine produced tocolytic effects by inhibiting both Ca^{2+}-dependent and Ca^{2+}-independent regulation of smooth muscle contraction pathways as well as by stimulating NO-cGMP pathway modulation. Both L-citrulline and L-arginine have been reported to be contained in watermelon flesh and rind. The findings in this chapter were the same as those of watermelon extracts described in Chapter IV. Thus, it is possible to speculate that the two amino acids might be the active contents of watermelon extracts that cause uterine relaxant in the present study.

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CHAPTER VI

EFFECTS OF WATERMELON (*CITRULLUS LANATUS*) EXTRACTS AND L-CITRULLINE ON NITRIC OXIDE

6.1 Abstract

Watermelon (*Citrullus lanatus*) is rich in L-citrulline and L-arginine; the contents that play an important role in the production of the potent vasodilator, nitric oxide (NO). Thus, the aim of this study was to clarify whether the tocolytic effects of watermelon extracts and L-citrulline, as shown in previous chapters, were due to NO-cGMP pathway modulation. This was done in isolated myometrial strips and the combination effects of watermelon extracts and L-citrulline measured. The contractile responses were recorded isometrically with a force transducer. The results revealed that the tocolytic effects of watermelon extracts and L-citrulline partially inhibited by L-NAME, a non-selective NO synthase, LY 83583, an inhibitor of soluble guanylate cyclase, and tetraethylammonium chloride, an inhibitor of calcium-activated potassium (K_{Ca}) channels. The combination of watermelon extracts and L-citrulline produced an additive effect on spontaneous contraction and the contractions induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), oxytocin, and potassium chloride solution (KCl). These findings suggest that the tocolytic effects of watermelon extracts and L-citrulline were via NO-cGMP signaling pathway and the activation of K_{Ca} channels. Moreover,

watermelon extracts and L-citrulline can interact additionally, indicating that the inhibitory effects of watermelon extracts may be due to L-citrulline.

6.2 Introduction

There is increasing evidence that nitric oxide (NO) has an important role in myometrial function (Yallampalli, Dong, Gangula and Fang, 1998). In living cells, NO is synthesized from L-arginine through the catalytic action of NO synthase (NOS). Three types of NOSs have been isolated and identified, including eNOS, nNOS, and iNOS. It has been reported that both eNOS and iNOS can be detected from non-pregnant rats (Yallampalli et al., 1998). However, nNOS was undetectable. Several investigations have indicated that NO production is increased during pregnancy and markedly decreased during spontaneous and induced preterm labor in rats (Yallampalli, Izumi, Byam-Smit and Garfield, 1994). However, there were no significant changes in the myometrium of pregnant and non-pregnant woman (Buhimschi, Yallampalli, Dong and Garfield, 1995), suggesting that NO-dependent uterine relaxation may depend on the species and the stages of menstrual period or gestation (Demirkoprulu et al., 2005). The formation of NO is inhibited by analogues of L-arginine such as N^G-nitro-L-arginine (L-NMMA), as well as aminoguanidine (Yallampalli et al., 1998).

NO diffuses into smooth muscle to activate soluble guanylate cyclase (sGC), leading to the activation of guanosine 3',-5'-cyclic monophosphate (cGMP) levels and the relaxation of smooth muscle (Carvajal, Germain, Huidobro-Toro and Weiner, 2000). Moreover, NO may also stimulate other enzymes that may be associated with

relaxation (Carvajal et al., 2000). It has been demonstrated that methylene blue and LY 83583 are generally used as sGC inhibitors to verify the involvement of NO-cGMP-dependent pathway modulation in many physiological processes (Kuenzli, Iain, Buxton and Bradley, 1998). It was indicated that activated cGMP leads to stimulate cGMP-dependent protein kinase (PKG), and may also activate cAMP-dependent protein kinase (PKA) (Carvajal et al., 2000). The phosphorylation of specific target proteins by PKG and PKA produces relaxation of various smooth muscles, including the myometrium (Carvajal et al., 2000). It has been reported that the relaxant effects of L-arginine on uterine smooth muscle appear to be due to NO-cGMP-dependent and NO-cGMP-independent pathways (Kuenzli et al., 1998; Norman, 1996). There is evidence that NO-cGMP-independent mechanisms are mostly associated with a direct action of NO on calcium-activated potassium (K_{Ca}) channels and L-type Ca^{2+} channels (Bradley, Buxton, Barber, Mcgaw and Bradley, 1998; Carvajal et al., 2000; Norman, 1996).

The clinical use of combinations of the component individual or mixtures of herbs has been found to be effective in increases of their therapeutic efficacy (Williamson, 2001). The aim of the combination is to achieve an additive interaction, yielding a sufficient therapeutic effect with low doses and reducing the prevalence of serious diseases (Williamson, 2001). As discussed in Chapter IV regarding the inhibitory effects of watermelon extracts were similar to those produced by L-citrulline and L-arginine (as shown in Chapter V). In addition, it was indicated that both fruit flesh and rind of watermelon are an excellent source of L-citrulline and L-arginine (Tlili, Hdider, Lenucci, Ilahy, Jebari and Dalessandro, 2011). It was of interest to verify whether the effects of the extracts were due to these compounds.

Therefore, the aims of this chapter were 1) to investigate whether the tocolytic effects of watermelon extracts and L-citrulline were produced by the NO-cGMP pathway modulation and 2) to examine whether the effects of watermelon extracts were due to L-citrulline.

6.3 Materials and Methods

6.3.1 Myometrial Tissue Preparations and Measurements of Tension

Myometrial tissue preparations were dissected and force measurements measured as those described in Chapter II (Sections 2.2.3. and 2.2.4, respectively).

6.3.2 Experimental Procedures

6.3.2.1 Effects on Endogenous Nitric Oxide Pathway

To investigate whether the tocolytic effects of watermelon extracts and L-citrulline were dependent upon endogenous NO activity, L-NAME (100 μM), a non-specific NOS inhibitor, was used. In the experiment, L-NAME was applied for 30 min and then watermelon extract or L-citrulline added, in the continued presence of L-NAME. In addition, the experiments were done the other way round. To do so, the extract or L-citrulline was applied for 30 min and then L-NAME added, in the continued presence of the extract or L-citrulline.

6.3.2.2 Effects on Guanylate Cyclase Pathway

To investigate whether the tocolytic effects of watermelon extracts and L-citrulline were through the sGC pathway, LY 83583 (1 μM), a specific sGC inhibitor, was used. In the experiment, LY 83583 was applied for 30 min and then watermelon extract or L-citrulline added, in the continued presence of LY 83583. In addition, the experiments were done the other way round. To do so, the extract or L-citrulline was applied for 30 min and then LY 83583 added, in the continued presence of the extract or L-citrulline.

6.3.2.3 Effects on Calcium-Activated Potassium Channels

To investigate whether the tocolytic effects of watermelon extracts and L-citrulline were dependent upon the role of K_{Ca} channels, tetraethylammonium chloride (TEA, 5 mM), a K_{Ca} channel inhibitor, was used. In the experiment, TEA was applied for 30 min and then watermelon extract or L-citrulline added, in the continued presence of TEA. In addition, the experiments were done the other way round. To do so, the extract or L-citrulline was applied for 30 min and then TEA added, in the continued presence of the extract or L-citrulline.

6.3.2.4 Effects of the Combinations of Watermelon Extracts and L-Citrulline

To investigate whether the inhibitory effects of watermelon extracts were due to L-citrulline, 64 μM L-citrulline was used. In the experiment, flesh or rind extract was applied for 30 min and then L-citrulline added, in the continued presence of flesh

or rind extract. In addition, the experiments were done the other way round. To do so, L-citrulline was applied for 30 min and then flesh or rind extract added, in the continued presence of L-citrulline. In some experiments, the effects of the combinations of watermelon extracts and L-citrulline on uterine contractions-induced by $PGF_{2\alpha}$, oxytocin, and KCl were also studied.

6.3.3 Chemicals and Physiological Solutions

All chemicals were purchased from Sigma®, Singapore. L-citrulline was dissolved in Krebs' solution. L-NAME, a non-specific NOS inhibitor; (N^G-nitroarginine methyl ester), was dissolved in distilled water and used at the concentration of 100 μM (Yallampalli, Garfield and Byam-Smith, 1993). LY 83583, a sGC inhibitor was dissolved in distilled water and used at concentration of 1 μM (Kuenzli et al., 1998). TEA, the calcium-dependent potassium channels inhibitor; (tetaethylamonium chloride), was dissolved in distilled water and used at concentration of 5 mM (Kupittayanant, Luckas and Wray, 2002). Prostaglandin $F_{2\alpha}$, ($PGF_{2\alpha}$-tris; (5Z,9_α,11_α,13E,15)-9,11,15-trihydroxyprosta-5,13-dienoic acid tris salt), was dissolved in the absolute ethanol and used at the concentration of 1 μM (Buddhakala, Talubmook, Sriyotha, Wray and Kupittayanant, 2008). Oxytocin was dissolved in distilled water and used at concentration of 10 nM (Kupittayanant et al., 2002). A KCl (40 mM) Krebs' solution was made by isoosmotic replacement of sodium chloride (Noble and Wray, 2002). The physiological Krebs' solution (pH 7.4) contained the following (mM): NaCl 154.0, KCl 5.4, $MgSO_4$ 1.2, glucose 8.0, $CaCl_2$ 2.0, and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 10.0.

6.3.4 Preparation of Watermelon Flesh and Rind Extracts

As described in Chapter II (Section 2.1.2), stocks of watermelon extracts were kept at -20°C. Fruit flesh or rind extract was dissolved in Krebs' solution just before use.

6.3.5 Statistical Analysis

The data were analyzed using Microcal Origin Software. The integral force was used as the parameter of contraction. Results were expressed as percentages of control contractions (i.e. the control is 100%). Throughout, data are presented as mean ± S.E.M. and "n" represents the number of samples, each one from a different animal. Significances were tested using appropriate *t* tests. The P value < 0.05 was taken to be significant.

6.4 Results

6.4.1 Effects of Watermelon Flesh and Rind Extracts on Rat Uterine Contraction in the Presence of NOS Inhibitor

Watermelon Flesh Extract

It is well documented that watermelon contains a high level of L-citrulline and L-arginine (Tlili et al., 2011). Clinical data indicated that watermelon supplementation produced a marked decrease in blood pressure in hypertensive men (Figueroa, Sanchez-Gonzalez, Perkins-Veazie and Arjmandi, 2011). It is believed that

L-citrulline and L-arginine found in watermelon may be served as NO precursors to mediate vasodilation in human subjects in this report (Figueroa et al., 2011). To investigate whether the inhibitory effects of watermelon flesh extract were involved with NO, the uterine strip was incubated with L-NAME (100 μM), a non-selective NOS inhibitor, and the effects of the watermelon flesh extract studied. The result showed that L-NAME markedly altered the inhibitory effects of watermelon flesh extract (Figure 6.1A). The amplitude and the mean integral force produced by the combination of the extract and L-NAME were 87.90 ± 4.83% and 82.16 ± 6.85% ($P > 0.05$), respectively when compared with spontaneous contraction (100%, n = 6). When L-NAME was added after the flesh extract, it reversed the inhibitory effects of flesh extract, but the amplitude of contractions did not return to spontaneous phasic contractions. The samples of experimental traces are shown in Figure 6.1 and data summarized in Table 6.1.

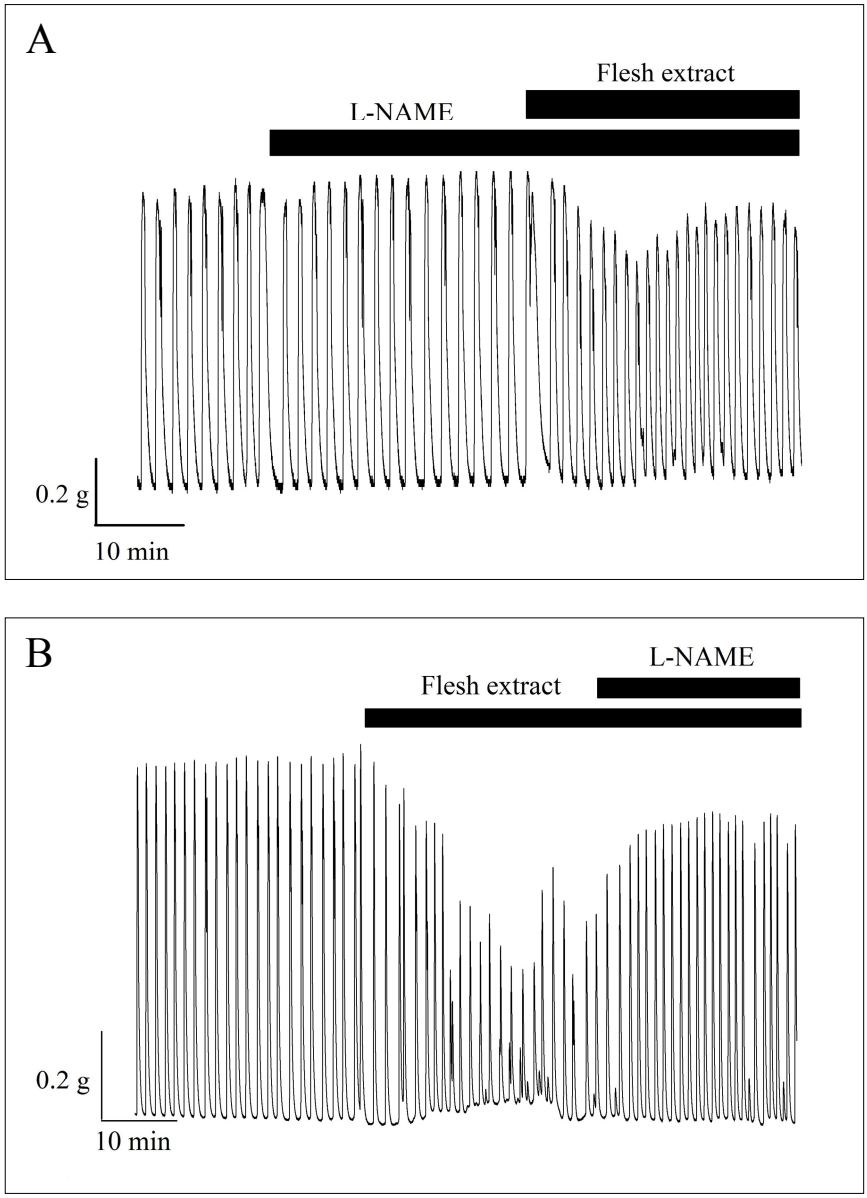

Figure 6.1 The effects of watermelon flesh extract on uterine contraction in the presence of NOS inhibitor. L-NAME (100 μM) was added before (A) and after (B) watermelon flesh extract (6 mg/mL) (n = 6 for each).

Table 6.1 The effects of watermelon flesh extract on uterine contraction in the presence of nitric oxide synthase inhibitor.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
Watermelon flesh (after)				
Control	100	100	100	6
L-NAME	104.14 ± 3.21	101.29 ± 4.43	82.16 ± 6.85	6
L-NAME + watermelon flesh	87.90 ± 4.83	113.63 ± 2.24*	84.99 ± 6.66	6
Watermelon flesh (before)				
Control	100	100	100	6
Watermelon flesh	53.70 ± 8.22*	114.50 ± 4.76*	60.69 ± 7.04*	6
Watermelon flesh + L-NAME	79.88 ± 5.10*	80.34 ± 2.40*	109.43 ± 4.62	6

The *P*-values for amplitude, frequency and AUC of L-NAME treated are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

Watermelon Rind Extract

The effects of watermelon rind extract on rat uterine contraction in the presence of L-NAME were also examined. As can be seen in Figure 6.2A, the application of rind extract in the continued presence of L-NAME did not change any force (85.59 ± 7.09%, $P > 0.05$, n = 6, compared with spontaneous contraction). The application of L-NAME in the continued presence of the rind extract produced an increase in force. The amplitude and the mean integral force produced by the combination of rind extract and L-NAME measured after 10 min of application were 77.13 ± 4.43% ($P < 0.05$) and 99.48 ± 7.63% ($P > 0.05$), respectively compared with spontaneous contraction (100%, n = 7). The samples of experimental traces are shown in Figure 6.2 and data summarized in Table 6.2.

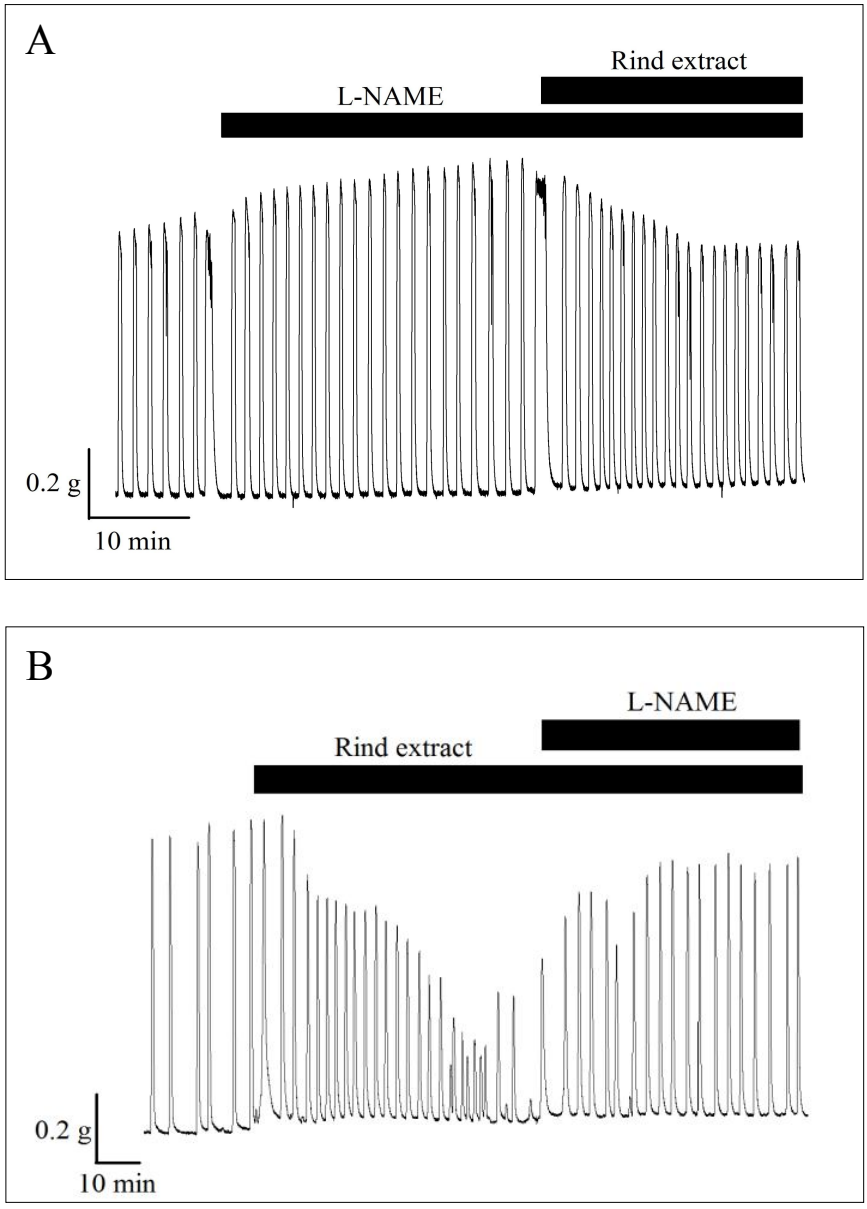

Figure 6.2 The effects of watermelon rind extract on uterine contraction in the presence of NOS inhibitor. L-NAME (100 μM) was added before (A, n = 6) and after (B, n = 7) watermelon rind extract (5 mg/mL).

Table 6.2 The effects of watermelon rind extract on uterine contraction in the presence of nitric oxide synthase inhibitor.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
Watermelon rind (after)				
Control	100	100	100	6
L-NAME	102.18 ± 8.75	95.13 ± 3.12	102.99 ± 5.94	6
L-NAME + watermelon rind	85.59 ± 7.09	114.24 ± 4.69*	93.79 ± 9.62	6
Watermelon rind (before)				
Control	100	100	100	7
Watermelon rind	52.68 ± 3.25*	116.80 ± 3.96*	65.27 ± 4.07*	7
Watermelon rind + L-NAME	77.13 ± 4.43*	131.66 ± 8.22*	99.48 ± 7.63	7

The *P*-values for amplitude, frequency and AUC of L-NAME treated are significantly different from the control ($^{*}P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

6.4.2 Effects of L-Citrulline on Rat Uterine Contraction in the Presence of NOS Inhibitor

To investigate whether the tocolytic effects of L-citrulline were due to NO, the uterine strip was pre-treated with L-NAME and the effects of L-citrulline studied. As can be seen in Figure 6.3A, pre-treatment of the uterine strip with L-NAME abolished the inhibitory effects of L-citrulline on rat uterine contraction. The amplitude and the mean integral force produced by the combination of L-NAME and L-citrulline measured after 10 min of the application were 89.96 ± 4.12% and 94.54 ± 9.68% ($P > 0.05$), respectively when compared with spontaneous contraction (100%, n = 5). The addition of L-NAME in the continued presence of L-citrulline reversed the inhibitory effects of this amino acid. The samples of experimental traces are shown in Figure 6.3 and data summarized in Table 6.3.

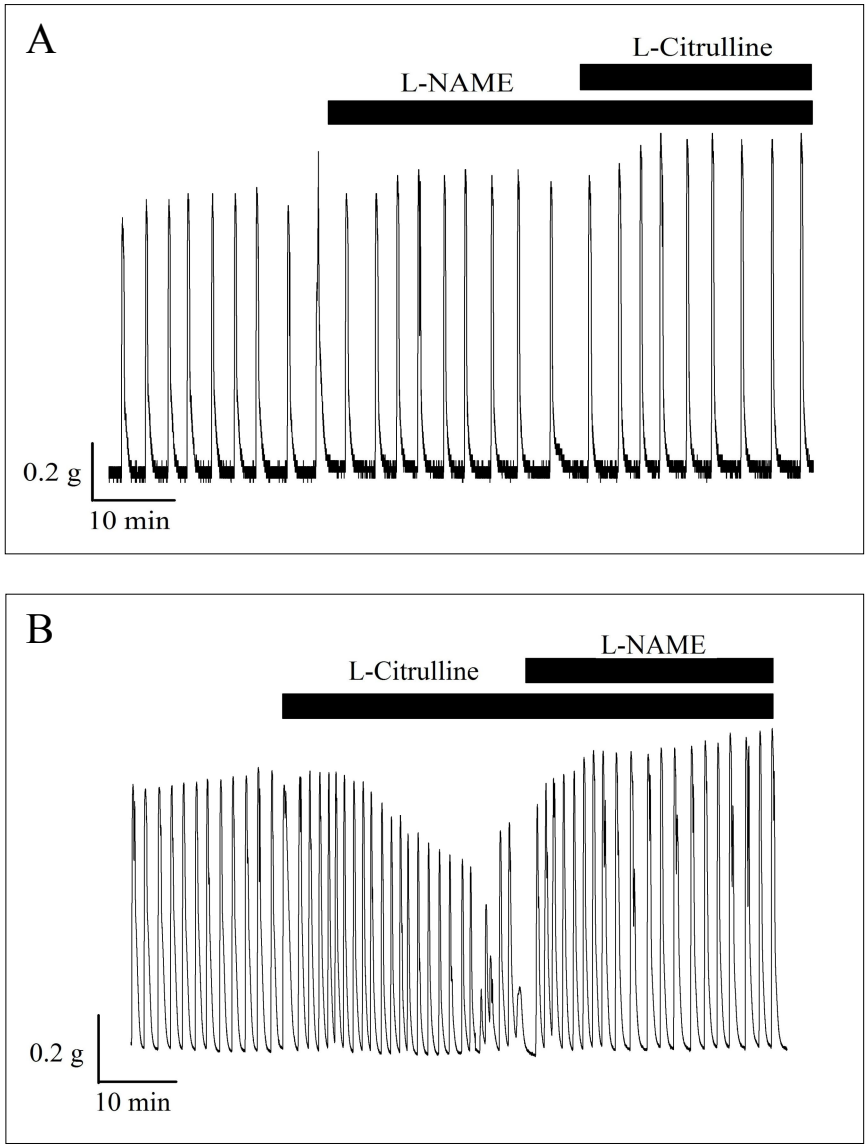

Figure 6.3 The effects of L-citrulline on uterine contraction in the presence of NOS inhibitor. L-NAME (100 μM) was added before (A) and after (B) L-citrulline (64 μM) (n = 5 for each).

Table 6.3 The effects of L-citrulline on uterine contraction in the presence of nitric oxide synthase inhibitor.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
L-citrulline (after)				
Control	100	100	100	5
L-NAME	106.87 ± 3.06	88.60 ± 6.98	96.02 ± 7.59	5
L-NAME + L-citrulline	89.96 ± 4.12	82.35 ± 6.35	94.54 ± 9.68	5
L-citrulline (before)				
Control	100	100	100	5
L-citrulline	61.02 ± 7.94*	105.74 ± 5.21	49.02 ± 5.64*	5
L-citrulline + L-NAME	101.53 ± 8.27	96.19 ± 6.66	90.89 ± 6.66	5

The *P*-values for amplitude, frequency and AUC of L-NAME treated are significantly different from the control ($^{*}P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

6.4.3 Effects of Watermelon Flesh and Rind Extracts on Rat Uterine Contraction in the Presence of Soluble Guanylate Cyclase Inhibitor

Watermelon Flesh Extract

It has been reported that NO exerts its relaxant effect through the activation of sGC, thereby increasing cGMP levels (Carvajal et al., 2000; Norman, 1996). cGMP stimulates PKG to regulate many physiological functions (Carvajal et al., 2000; Norman, 1996). It was suggested that activated PKG phosphorylates several key target proteins, including ion channels, ion pumps, and receptors (Carvajal et al., 2000; Norman, 1996). Phosphorylation of these target proteins decrease intracellular Ca^{2+} $[Ca^{2+}]_i$ and leads to relaxation of smooth muscle (Carvajal et al., 2000; Norman, 1996). To test whether the tocolytic effects of watermelon flesh extract were associated with the production of cGMP, LY 83583, the sGC inhibitor, was used. As can be seen in Figure 6.4A, LY 83583 (1 μM) applied to uterine tissue did not cause any change in force. The application of watermelon flesh (6 mg/mL) extract in the presence of LY 83583 produced a marked decrease in the contractile tension. The amplitude and the mean integral force of contractions produced by the combination of the flesh extract and LY 83583 measured after 10 min application were 67.28 ± 3.55% and 81.08 ± 7.86%, ($P < 0.05$) respectively when compared with spontaneous contraction (100%, n = 6). When LY 83583 was added after the flesh extract, it produced a small increase in phasic contractions (Figure 6.4B). The amplitude and the mean integral force produced by the combination of the flesh extract and LY 83583 measured after 10 min application were 69.14 ± 6.14% and 71.02 ± 8.94%, ($P < 0.05$) respectively when compared with spontaneous contraction

(100%, n = 8). The samples of experimental traces are shown in Figure 6.4 and data summarized in Table 6.4.

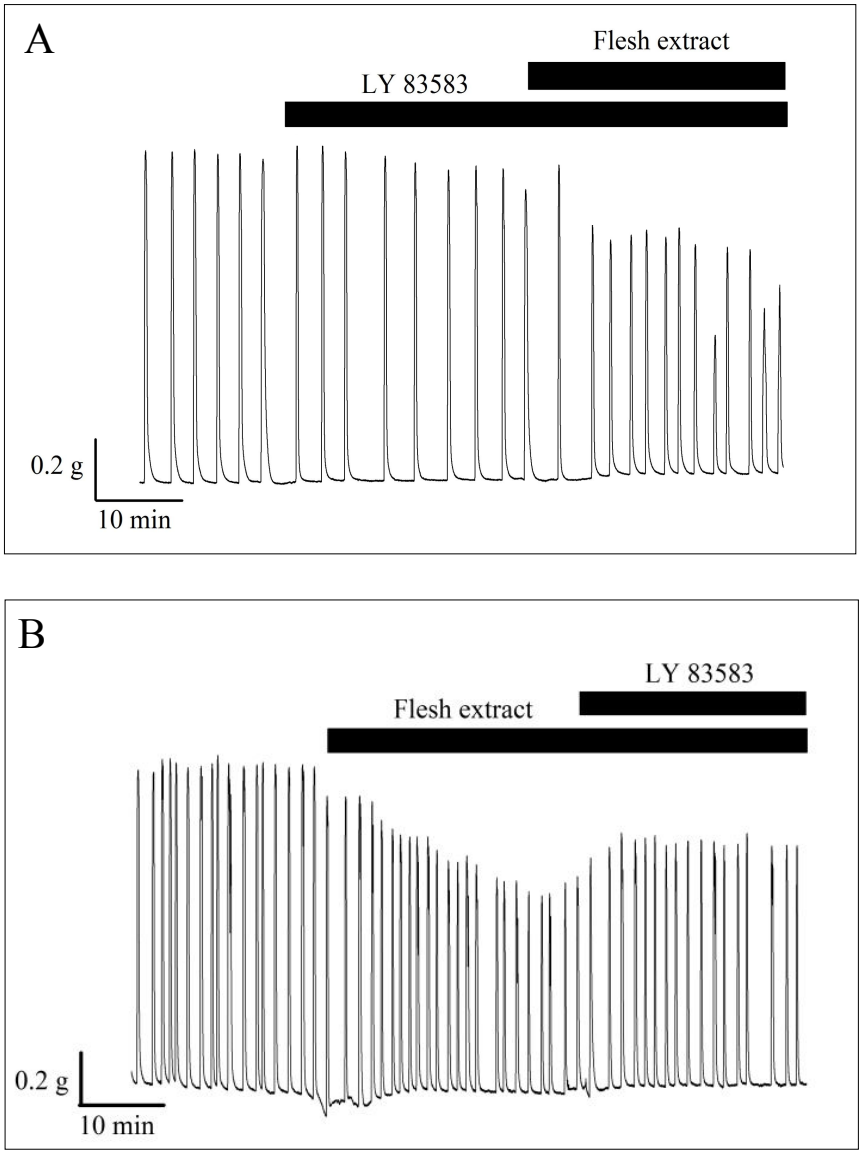

Figure 6.4 The effects of watermelon flesh extract on uterine contraction in the presence of soluble guanylate cyclase inhibitor. LY 83583 (1 μM) was added before (A, n = 6) and after (B, n = 8) watermelon flesh extract (6 mg/mL).

Table 6.4 The effects of watermelon flesh extract on uterine contraction in the presence of soluble guanylate cyclase inhibitor.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
Watermelon flesh (after)				
Control	100	100	100	6
LY 83583	109.10 ± 7.73	93.75 ± 4.26	95.59 ± 5.17	6
LY 83583 + watermelon flesh	67.28 ± 3.55*	111.57 ± 5.93	81.08 ± 7.86*	6
Watermelon flesh (before)				
Control	100	100	100	8
Watermelon flesh	49.73 ± 5.89*	114.79 ± 5.64	59.50 ± 6.29*	8
Watermelon flesh + LY 83583	69.14 ± 6.14*	90.83 ± 7.86	71.02 ± 8.94*	8

The *P*-values for amplitude, frequency and AUC of LY 83583 treated are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

Watermelon Rind Extract

To verify the possible contribution of cGMP to the tocolytic effects of watermelon rind extract, LY 83583 was added to the organ bath. 30 min later, watermelon rind extract (5 mg/mL) was then added. The result showed that the amplitude (73.03 ± 4.14%) and the mean integral force (75.15 ± 8.94%) of the contraction were significantly decreased when compared with spontaneous contraction (100%, n = 9) (Figure 6.5A). Addition of LY 83583 to uterine tissue in the presence the watermelon rind extract partially reversed the tocolytic effects of the plant extract (Figure 6.5B). The amplitude of the contraction produced by the combination of the rind extract and LY 83583 measured after 10 min application was 76.13 ± 5.03% ($P < 0.05$), when compared with spontaneous contraction (100%, n = 6). The samples of experimental traces are shown in Figure 6.5 and data summarized in Table 6.5.

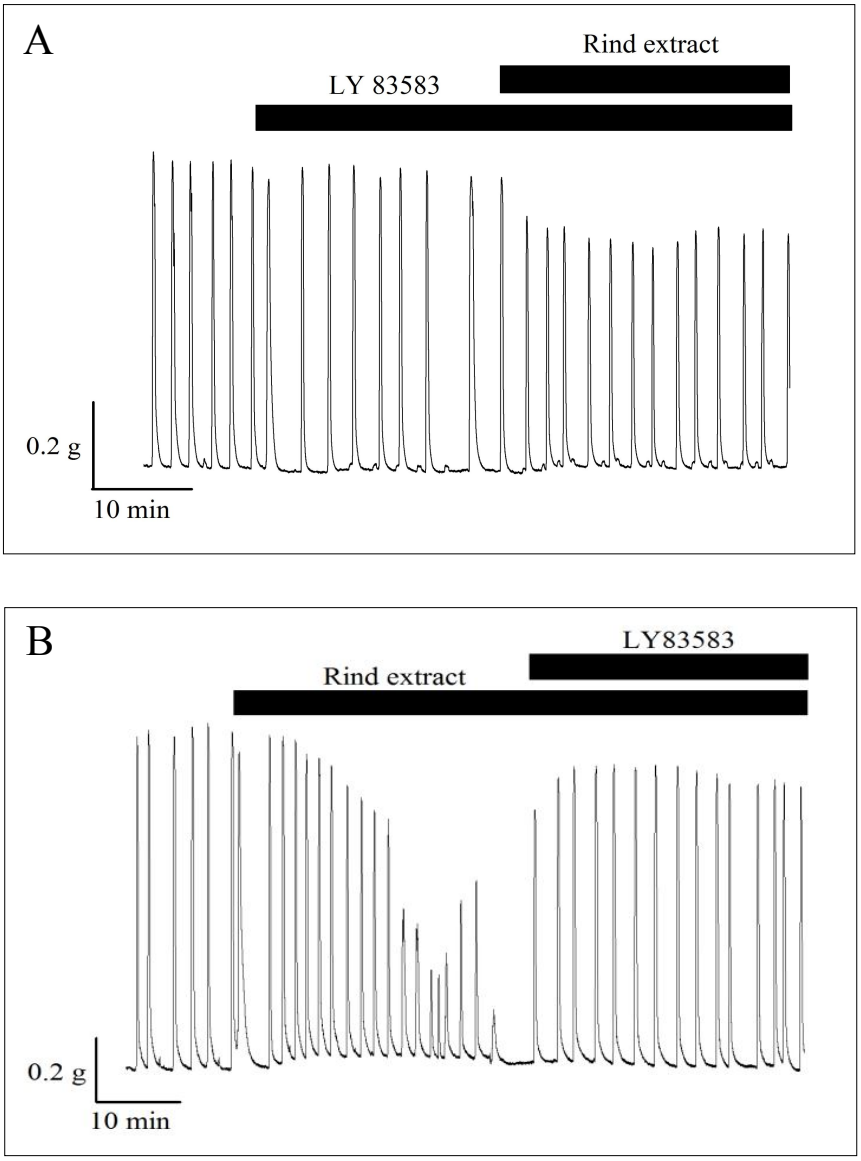

Figure 6.5 The effects of watermelon rind extract on uterine contraction in the presence of soluble guanylate cyclase inhibitor. LY 83583 (1 µM) was added before (A, n = 9) and after (B, n = 6) watermelon rind extract (5 mg/mL).

Table 6.5 The effects of watermelon rind extract on uterine contraction in the presence of soluble guanylate cyclase inhibitor.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
Watermelon rind (after)				
Control	100	100	100	9
LY 83583	108.73 ± 5.40	92.61 ± 3.63	93.93 ± 7.51	9
LY 83583 + watermelon rind	73.03 ± 4.14*	104.14 ± 4.50	75.15 ± 8.94	9
Watermelon rind (before)				
Control	100	100	100	6
Watermelon rind	52.68 ± 3.25*	118.33 ± 2.47*	58.13 ± 7.12*	6
Watermelon rind + LY 83583	76.13 ± 5.03*	119.66 ± 5.48*	91.26 ± 3.65	6

The *P*-values for amplitude, frequency and AUC of LY 83583 treated are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

6.4.4 Effects of L-Citrulline on Rat Uterine Contraction in the Presence of Soluble Guanylate Cyclase Inhibitor

To define the role of the involvement of cGMP in the inhibitory effects of L-citrulline, the uterine strips were pre-incubated with LY 83583 and then L-citrulline (64 μM) added. The amplitude (94.89 ± 4.67%) and the mean integral force (82.73 ± 8.96%) of the contractions did not change when compared with spontaneous phasic contractions (100%, n = 5, Figure 6.6A). The application of L-citrulline to spontaneous contraction reduced the uterine force. The addition of LY 83583 in the continued presence of L-citrulline did not affect the amplitude of the contraction (90.43 ± 9.39%, n = 8, compared with spontaneous contraction, 100%). The samples of experimental traces are shown in Figure 6.6 and data summarized in Table 6.6.

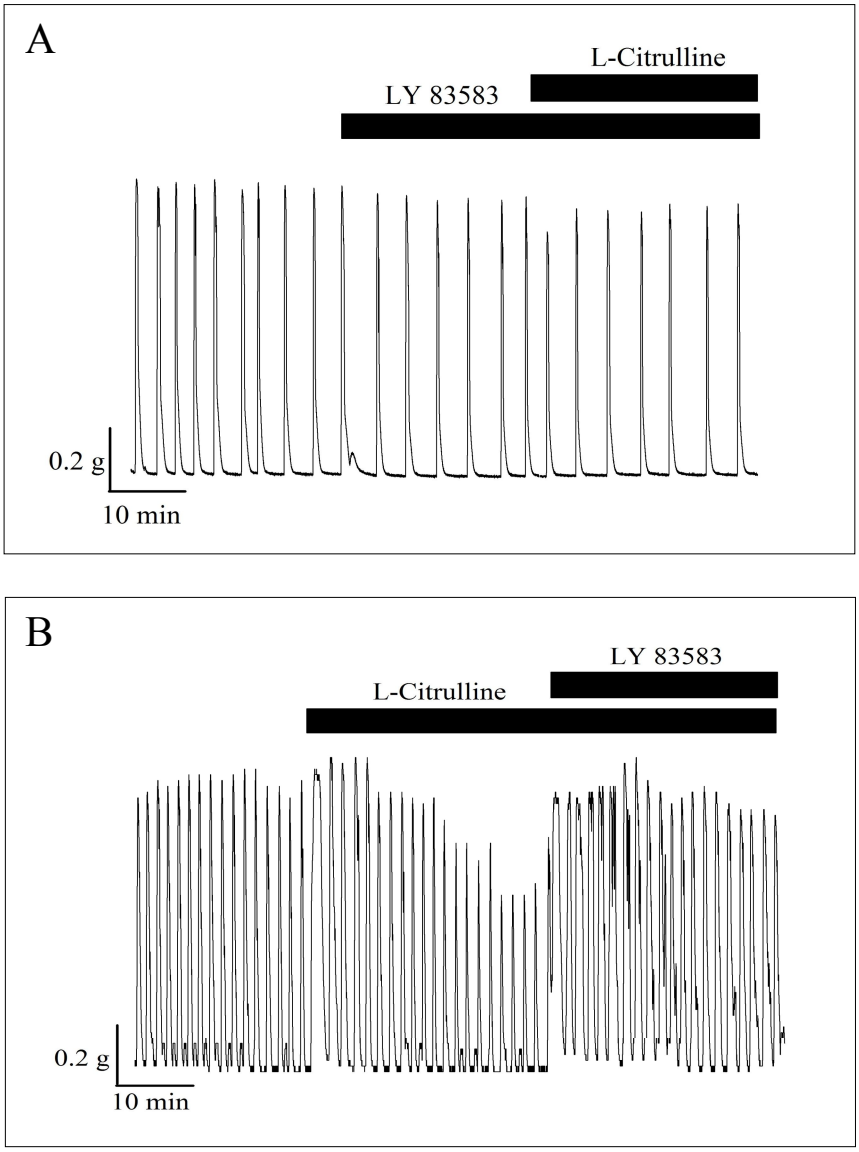

Figure 6.6 The effects of L-citrulline on uterine contraction in the presence of soluble guanylate cyclase inhibitor. LY 83583 (1 μM) was added before (A, n = 5) and after (B, n = 8) L-citrulline (64 μM).

Table 6.6 The effects of L-citrulline on uterine contraction in the presence of soluble guanylate cyclase inhibitor.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
L-citrulline (after)				
Control	100	100	100	5
LY 83583	103.61 ± 3.86	108.22 ± 5.24	101.38 ± 7.90	5
LY 83583 + L-citrulline	94.89 ± 4.67	106.25 ± 9.93	82.73 ± 8.96	5
L-citrulline (before)				
Control	100	100	100	8
L-citrulline	69.86 ± 7.84*	108.85 ± 5.96	59.23 ± 6.90*	8
L-citrulline + LY 83583	90.43 ± 9.39	105.85 ± 3.58	86.73 ± 4.9*	8

The *P*-values for amplitude, frequency and AUC of LY 83583 treated are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

6.4.5 Effects of Watermelon Flesh and Rind Extracts on Rat Uterine Contraction in the Presence of Calcium-Activated Potassium Channel Inhibitor

Watermelon Flesh Extract

It has been revealed that the efflux of K^+ through K_{Ca} channels is directly associated with the level of Ca^{2+} (Carvajal et al., 2000). It was indicated that the opening of K_{Ca} channels leads to hyperpolarization of the cell membrane and inhibition of Ca^{2+} entry through voltage-gated Ca^{2+} channels (Carvajal et al., 2000). Decrease of the Ca^{2+} influx via the voltage-gated Ca^{2+} channels results in smooth muscle relaxation. It was suggested that phosphorylation of K_{Ca} channels by PKG also decrease smooth muscle tone (Carvajal et al., 2000; Norman, 1996). In addition, there is evidence that NO can directly stimulate K_{Ca} channels to mediate relaxation (Bolotina, Najibi, Palacino, Pagano and Cohen, 1994; Carvajal et al., 2000). This mechanism is usually referred to cGMP-independent pathway modulation of smooth muscle relaxation (Bolotina et al., 1994; Carvajal et al., 2000). Thus, it was of interest to gain insight into the molecular mechanism by which watermelon flesh extract activates K_{Ca} channels. To do so, TEA, the K_{Ca} channel inhibitor, was used in this study. The application of TEA (5 mM) to spontaneously active phasic contractions led to significant increases in the amplitude and the mean integral force of the contraction (Figure 6.7A). The addition of the flesh extract in the presence of TEA produced a small decrement of force (89.56 ± 5.41%, $P > 0.05$) when compared with spontaneous contraction (100%, n = 8). When TEA was added after the flesh extract, it partially reversed the tocolytic effects of watermelon extract (Figure 6.7B). The amplitude and

the mean integral force produced by the combination of the flesh extract and TEA measured after 10 min application were 82.90 ± 3.42% ($P < 0.05$) and 94.18 ± 6.85% ($P > 0.05$), respectively when compared with spontaneous contraction (100%, n = 7). The samples of experimental traces are shown in Figure 6.7 and data summarized in Table 6.7.

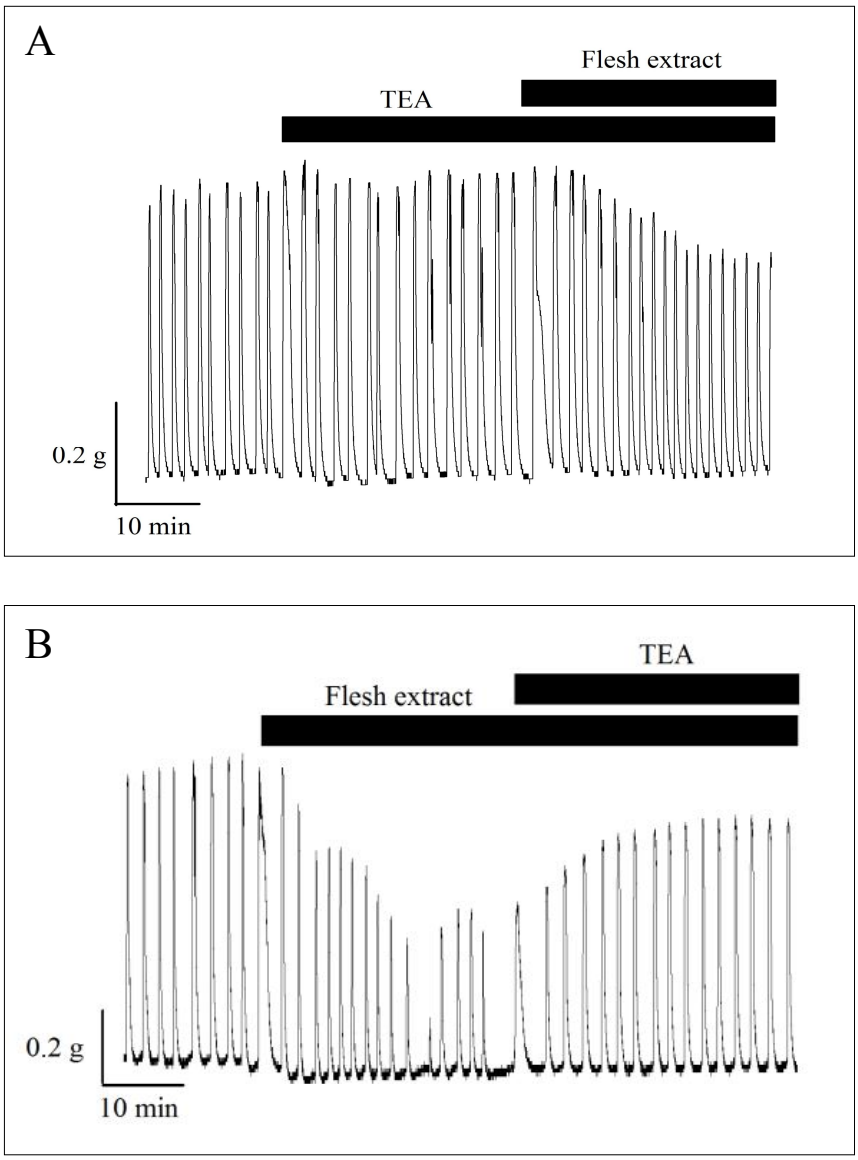

Figure 6.7 The effects of watermelon flesh extract on uterine contraction in the presence of calcium activated potassium channel inhibitor. TEA (5 mM) was added before (A, n = 8) and after (B, n = 7) watermelon flesh extract (6 mg/mL).

Table 6.7 The effects of watermelon flesh extract on uterine contraction in the presence of calcium-activated potassium channel inhibitor.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
Watermelon flesh (after)				
Control	100	100	100	8
TEA	108.78 ± 1.66	123.54 ± 2.84*	122.67 ± 5.62*	8
TEA + watermelon flesh	89.56 ± 5.41	118.75 ± 4.19*	90.12 ± 5.19	8
Watermelon flesh (before)				
Control	100	100	100	7
Watermelon flesh	66.19 ± 2.83*	114.49 ± 3.95	50.22 ± 3.82*	7
Watermelon flesh + TEA	82.90 ± 3.42*	116.92 ± 5.27	94.18 ± 6.85	7

The *P*-values for amplitude, frequency and AUC of TEA treated are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

Watermelon Rind Extract

The effects of watermelon rind extract on uterine contractions in the presence of K_{Ca} channel inhibitor were also studied. As can be seen in Figure 6.8A, addition of the rind extract in the continued presence of TEA produced a small decrease in contraction amplitude of the contraction to 89.90 ± 3.31% ($P > 0.05$), when compared with spontaneous contraction (100%, n = 8). When TEA was added after the addition of the rind extract, it caused an increase in force (Figure 6.8B). The amplitude and the mean integral force produced by the combination of watermelon rind extract and TEA measured after 10 min application were 92.36 ± 3.22% and 109.20 ± 6.37%, ($P > 0.05$) respectively when compared with spontaneous contraction (100%, n = 5). The samples of experimental traces are shown in Figure 6.8 and data summarized in Table 6.8.

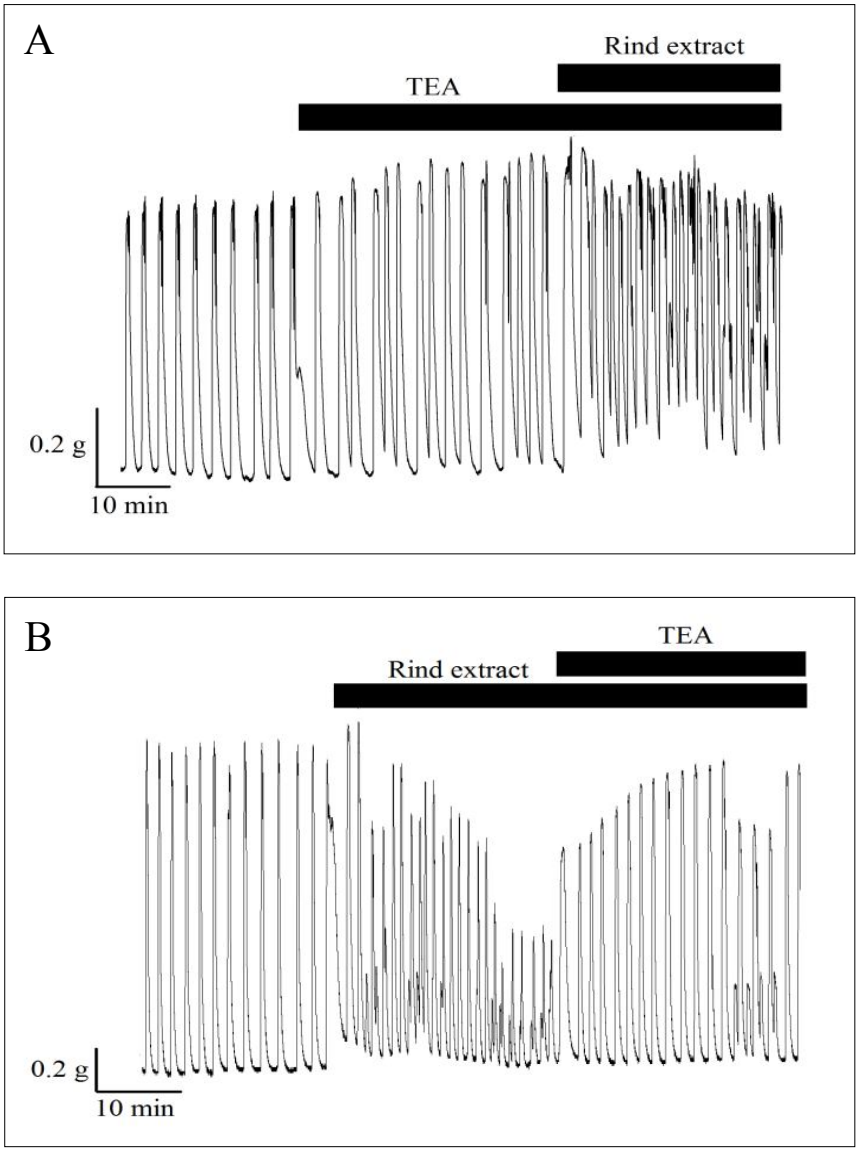

Figure 6.8 The effects of watermelon rind extract on uterine contraction in the presence of calcium activated potassium channel inhibitor. TEA (5 mM) was added before (A, n = 8) and after (B, n = 5) watermelon rind extract (5 mg/mL).

Table 6.8 The effects of watermelon rind extract on uterine contraction in the presence of calcium-activated potassium channel inhibitor.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
Watermelon rind (after)				
Control	100	100	100	8
TEA	114.90 ± 2.27*	116.99 ± 4.36*	124.99 ± 4.80*	8
TEA + watermelon rind	89.90 ± 3.31	122.17 ± 8.56*	113.09 ± 7.68*	8
Watermelon rind (before)				
Control	100	100	100	5
Watermelon rind	63.25 ± 3.39*	113.66 ± 4.16	67.39 ± 7.11*	5
Watermelon rind + TEA	92.36 ± 3.22	113.33 ± 5.65	109.20 ± 6.37	5

The *P*-values for amplitude, frequency and AUC of TEA treated are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

6.4.6 Effects of L-Citrulline on Rat Uterine Contraction in the Presence of Calcium-Activated Potassium Channel Inhibitor

As can be seen in Figure 6.9A, the application of L-citrulline in the presence of TEA produced a small decrease in contraction amplitude and the mean integral force to 89.07 ± 9.49% and 94.48 ± 5.67%, ($P > 0.05$) respectively, when compared with spontaneous contraction (100%, n = 6). When TEA was added after the application of L-citrulline, it produced an increase in uterine tension (Figure 6.9B). The amplitude and the mean integral force produced by the combination of L-citrulline and TEA measured after 10 min application were 94.42 ± 8.24% and 93.87 ± 6.92%, ($P > 0.05$) respectively when compared with spontaneous contraction (100%, n = 6). The samples of experimental traces are shown in Figure 6.9 and data summarized in Table 6.9.

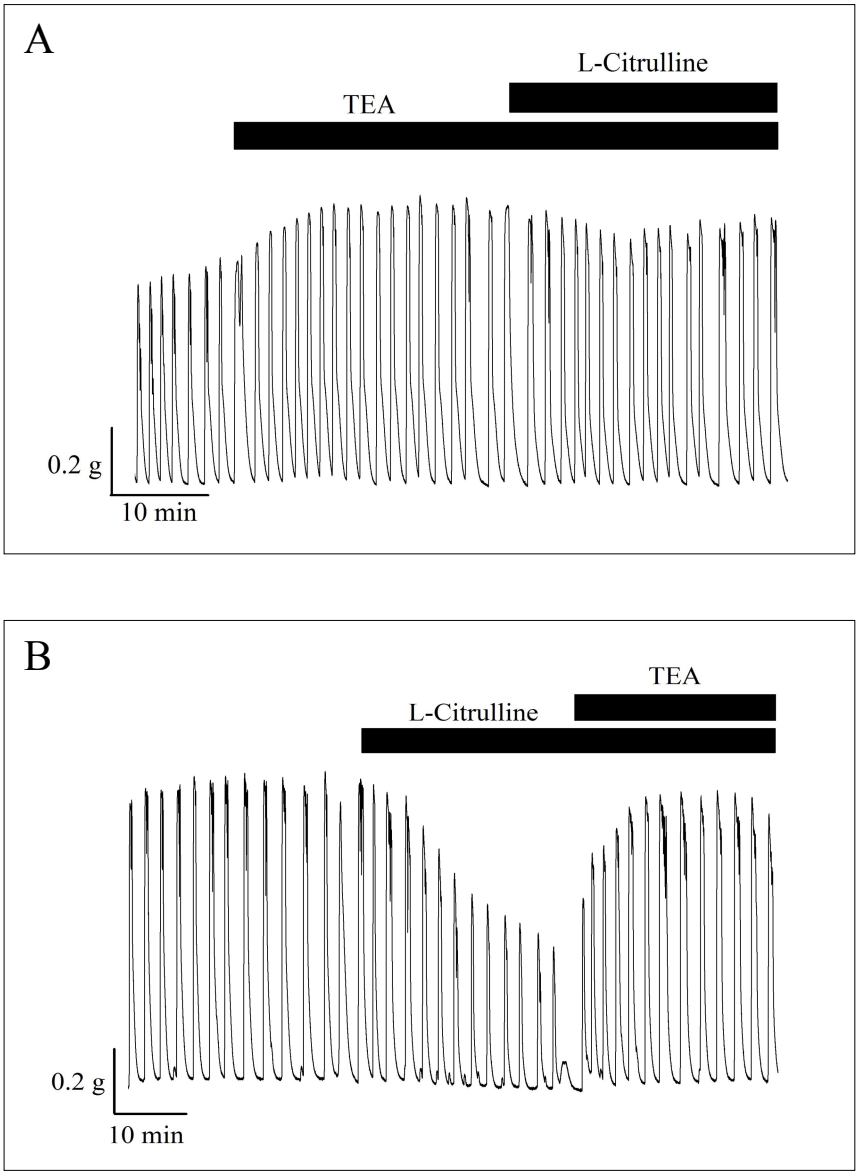

Figure 6.9 The effects of L-citrulline on uterine contraction in the presence of calcium activated potassium channel inhibitor. TEA (5 mM) was added before (A) and after (B) L-citrulline (64 μM) (n = 6 for each).

Table 6.9 The effects L-citrulline on uterine contraction in the presence of calcium-activated potassium channel inhibitor.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
L-citrulline (after)				
Control	100	100	100	6
TEA	116.37 ± 5.58*	118.85 ± 6.42*	126.75 ± 5.78*	6
TEA + L-citrulline	89.07 ± 9.49	105.57 ± 9.76	94.48 ± 5.67	6
L-citrulline (before)				
Control	100	100	100	6
L-citrulline	73.11 ± 7.85*	100.24 ± 8.83	61.44 ± 6.88*	6
L-citrulline + TEA	94.42 ± 8.24	115.15 ± 5.98*	93.87 ± 6.92	6

The *P*-values for amplitude, frequency and AUC of TEA treated are significantly different from the control ($^{*}P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

6.4.7 Effects of the Combinations of Watermelon Extracts and L-Citrulline on Spontaneous Contraction

As shown in Chapter IV, it is demonstrated that watermelon extracts have a potent tocolytic effect. L-citrulline is one of the major compositions found in watermelon (Rimando and Perkins-Veazie, 2005), it was worth determining whether the effects of watermelon extracts on uterine contraction were due to L-citrulline.

Watermelon Flesh Extract

Application of L-citrulline reduced the amplitude and the mean integral force of the contractions to 62.18 ± 4.45% and 65.49 ± 3.28%, ($P < 0.05$) respectively compared with spontaneous contraction (100%, n = 5). When watermelon flesh extract was added in the continued presence of L-citrulline, it produced a significant decrease in the amplitude (35.81 ± 8.02%) and the mean integral force (36.22 ± 4.22%) of contractions (Figure 6.10A). As shown in Figure 6.10B, when L-citrulline was added after an addition of watermelon flesh extract, it reduced the amplitude and the mean integral force of the contractions to 36.93 ± 2.54% and 37.29 ± 1.68%, ($P < 0.05$) respectively compared with spontaneous contraction (100%, n = 8). The samples of experimental traces are shown in Figure 6.10 and data summarized in Table 6.10.

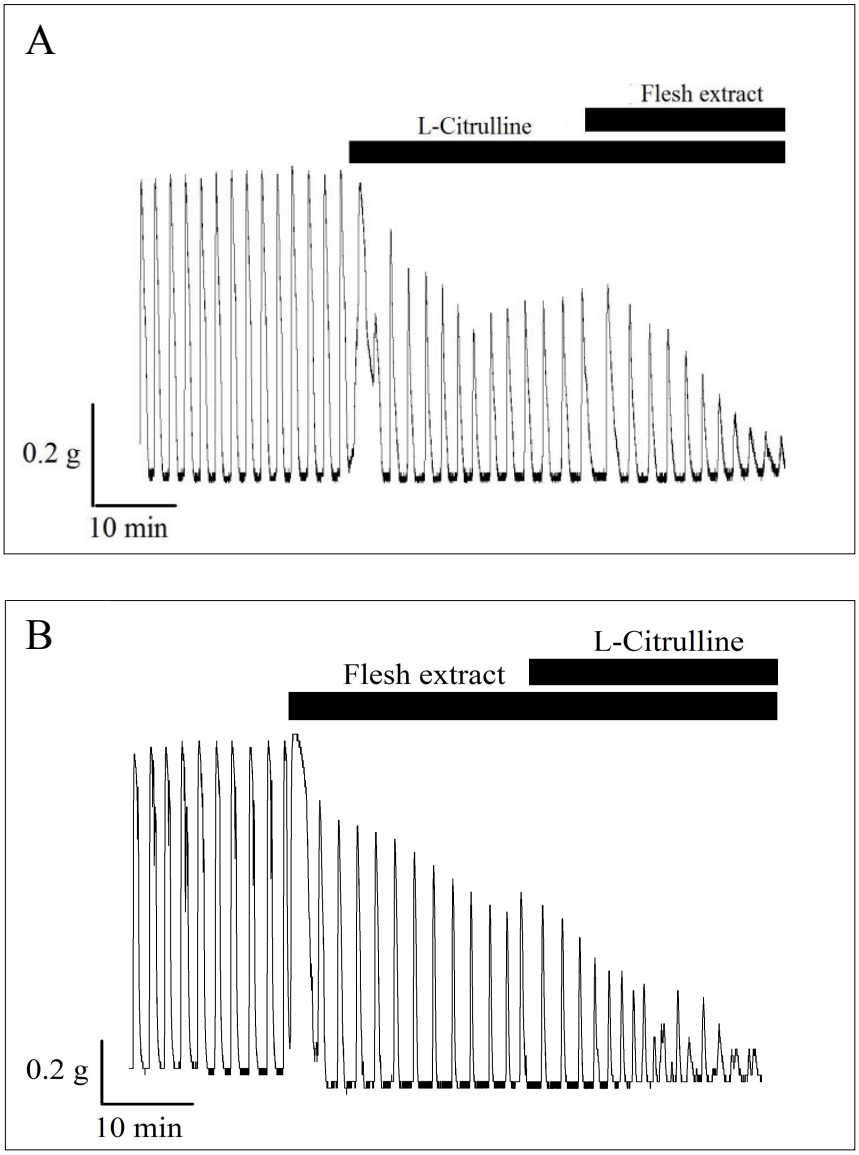

Figure 6.10 The effects of the combination of watermelon flesh extract and L-citrulline on spontaneous contractions. L-citrulline (64 μM) was added before (A, n = 5) and after (B, n = 8) watermelon flesh extract (6 mg/mL).

Table 6.10 The effects of the combination of watermelon flesh extract and L-citrulline on spontaneous contractions.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
Watermelon flesh (after)				
Control	100	100	100	5
L-citrulline	62.18 ± 4.45*	95.42 ± 8.09	65.49 ± 3.28*	5
L-citrulline + Watermelon flesh	35.81 ± 8.02*	101.25 ± 3.23*	36.22 ± 4.22*	5
Watermelon flesh (before)				
Control	100	100	100	8
Watermelon flesh	63.45 ± 6.64*	112.45 ± 2.94*	60.21 ± 3.58*	8
Watermelon flesh + L-citrulline	36.93 ± 2.54*	105.75 ± 4.95	37.29 ± 1.68*	8

The *P*-values for amplitude, frequency and AUC of watermelon flesh extract treated are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

Watermelon Rind Extract

As shown in Figure 6.11A, L-citrulline caused significant decreases in the amplitude and the mean integral force of the contractions. When the rind extract was applied in the continued presence of L-citrulline, it produced significant decreases in the amplitude (20.16 ± 2.20%) and the mean integral force (22.29 ± 3.24%) of the contractions, compared with spontaneous contraction (100%, n = 5). When L-citrulline was applied after the application of the rind extract, the amplitude and the mean integral force had fallen to 21.01 ± 5.63% and 22.27 ± 8.04%, ($P < 0.05$) respectively compared with spontaneous contraction (100%, n = 8). The samples of experimental traces are shown in Figure 6.11 and data summarized in Table 6.11.

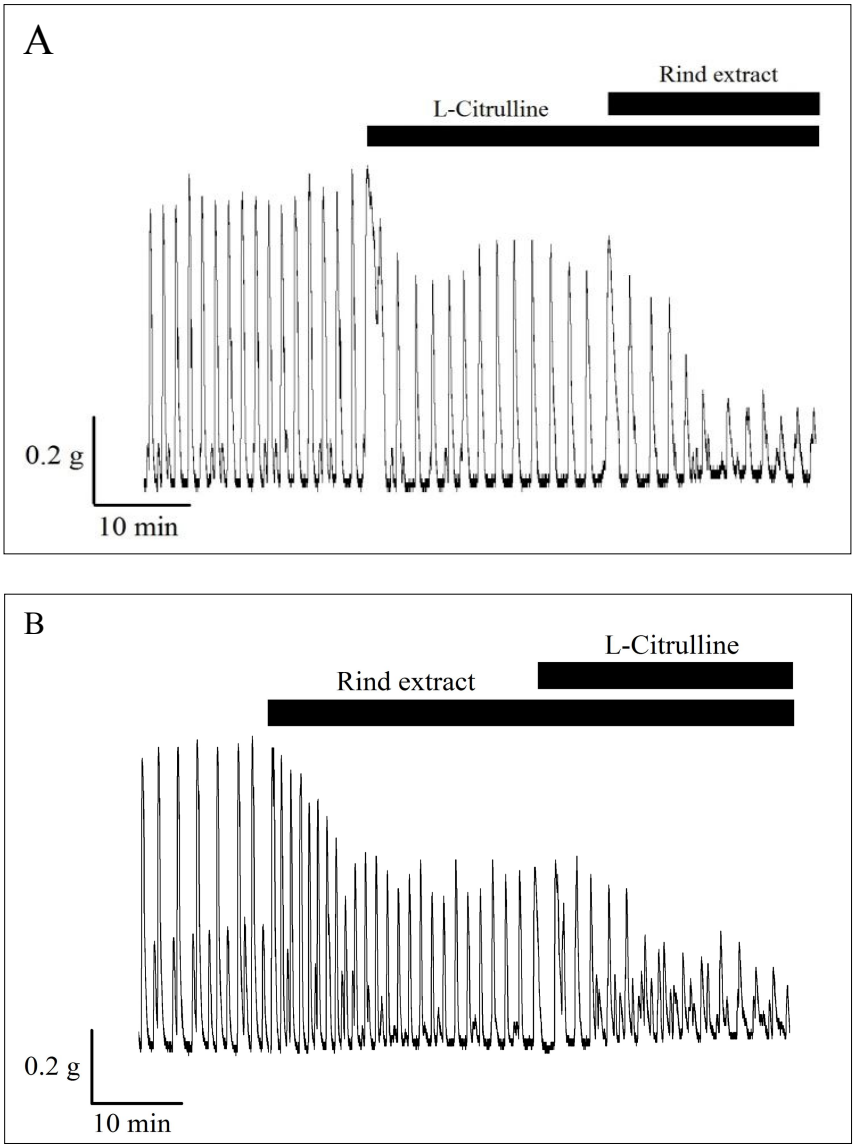

Figure 6.11 The effects of the combination of watermelon rind extract and L-citrulline on spontaneous contractions. L-citrulline (64 μM) was added before (A, n = 5) and after (B, n = 8) watermelon rind extract (5 mg/mL).

Table 6.11 The effects of the combination of watermelon rind extract and L-citrulline on spontaneous contractions.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
Watermelon rind (after)				
Control	100	100	100	5
L-citrulline	64.85 ± 1.28*	100.42 ± 5.89	62.44 ± 1.80*	5
L-citrulline + Watermelon rind	20.16 ± 2.20*	102.14 ± 6.98	22.29 ± 3.24*	5
Watermelon rind (before)				
Control	100	100	100	8
Watermelon rind	60.14 ± 6.37*	114.43 ± 2.92*	59.72 ± 3.60*	8
Watermelon rind + L-citrulline	21.01 ± 5.63*	98.18 ± 4.59	22.27 ± 8.04*	8

The *P*-values for amplitude, frequency and AUC of watermelon rind extract treated are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

6.4.8 Effects of the Combinations of Watermelon Extracts and L-Citrulline on $PGF_{2\alpha}$-Induced Uterine Contraction

Watermelon Flesh Extract and L-Citrulline

As can be seen in Figure 6.12A, application of $PGF_{2\alpha}$ (1 μM) to spontaneously contracting myometrium produced significant increases in the amplitude and the mean integral force of the contractions. Addition of the combination of the flesh extract (6 mg/mL) and L-citrulline (64 μM) to the myometrium in the continued presence of 1 μM $PGF_{2\alpha}$ produced a significant inhibition on both the amplitude and the mean integral force (n = 5). The samples of experimental traces are shown in Figure 6.12A and data summarized in Table 6.12.

Watermelon Rind Extract and L-Citrulline

As can be seen in Figure 6.12B, 1 μM $PGF_{2\alpha}$ significantly increased the amplitude, the frequency and the mean integral force of the contractions. Application of the combination of the rind extract (5 mg/mL) and L-citrulline (64 μM) to the myometrium in the continued presence of $PGF_{2\alpha}$ caused a significant inhibition on both the amplitude and the mean integral force (n = 5). The samples of experimental traces are shown in Figure 6.12B and data summarized in Table 6.12.

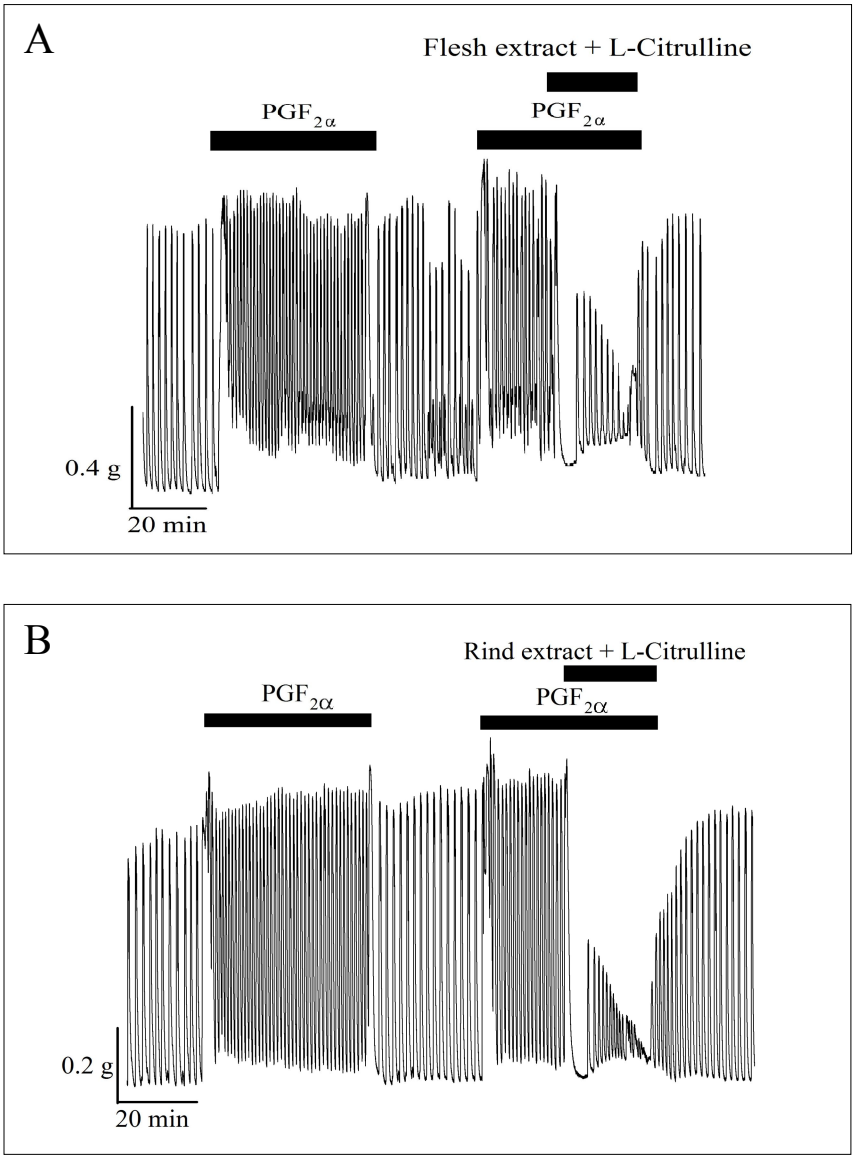

Figure 6.12 The effects of the combinations of watermelon extracts and L-citrulline on $PGF_{2\alpha}$-induced uterine contraction. The effects of the combination of watermelon flesh extract (6 mg/mL) and 64 µM L-citrulline (A) and the combination of watermelon rind extract (5 mg/mL) and 64 µM L-citrulline (B) on uterine contraction-induced by 1 µM $PGF_{2\alpha}$ are shown (n = 5 for each).

Table 6.12 The effects of the combinations of watermelon extracts and L-citrulline on $PGF_{2\alpha}$-induced uterine contraction.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
Watermelon flesh				
Control	100	100	100	5
$PGF_{2\alpha}$	120.09 ± 1.19*	125.44± 3.09*	122.44 ± 5.63*	5
$PGF_{2\alpha}$ + L-citrulline + watermelon flesh	26.29 ± 1.80*	112.01 ± 3.96*	28.02 ± 2.15*	5
Watermelon rind				
Control	100	100	100	5
$PGF_{2\alpha}$	123.25 ± 2.64*	126.86 ± 4.80*	125.81 ± 6.78*	5
$PGF_{2\alpha}$ + L-citrulline + watermelon rind	19.36 ± 4.78*	100.15 ± 3.58	20.73 ± 4.77*	5

The *P*-values for amplitude, frequency and AUC of 1 μM $PGF_{2\alpha}$ treated are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

6.4.9 Effects of the Combinations of Watermelon Extracts and L-Citrulline on Oxytocin-Induced Uterine Contraction

Watermelon Flesh Extract and L-Citrulline

As can be seen in Figure 6.13A, application of oxytocin (10 nM) to spontaneously contracting myometrium produced significant increases in the amplitude and the mean integral force of the contractions. Addition of the combination of the flesh extract (6 mg/mL) and L-citrulline (64 μM) to the myometrium in the continued presence of 10 nM oxytocin produced a significant inhibition on both the amplitude and the mean integral force (n = 5). The samples of experimental traces are shown in Figure 6.13A and data summarized in Table 6.13.

Watermelon Rind Extract and L-Citrulline

As can be seen in Figure 6.13B, the addition of oxytocin (10 nM) significantly increased the amplitude, the frequency and the mean integral force of the contractions. Application of the combination of the rind extract (5 mg/mL) and L-citrulline (64 μM) to the myometrium in the continued presence of oxytocin caused a significant inhibition on both the amplitude and the mean integral force (n = 5). The samples of experimental traces are shown in Figure 6.13B and data summarized in Table 6.13.

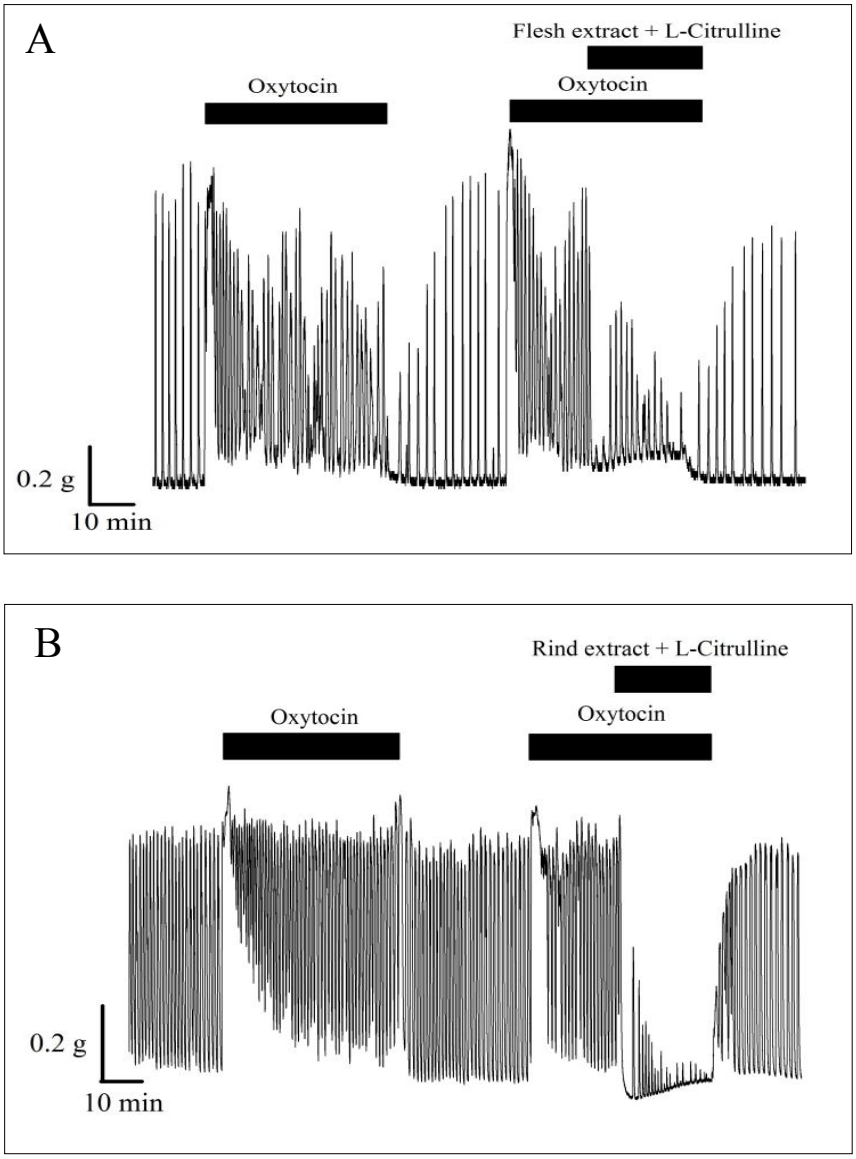

Figure 6.13 The effects of the combinations of watermelon extracts and L-citrulline on oxytocin-induced uterine contraction. The effects of the combination of watermelon flesh extract (6 mg/mL) and 64 μM L-citrulline (A) and the combination of watermelon rind extract (5 mg/mL) and 64 μM L-citrulline (B) on uterine contraction-induced by 10 nM oxytocin are shown (n = 5 for each).

Table 6.13 The effects of the combinations of watermelon extracts and L-citrulline on oxytocin-induced uterine contraction.

	Amplitude (% Mean ± S.E.M.)	Frequency (% Mean ± S.E.M.)	AUC (% Mean ± S.E.M.)	n
Watermelon flesh				
Control	100	100	100	5
Oxytocin	130.09 ± 2.19*	150.84± 8.09*	152.44 ± 6.80*	5
Oxytocin + L-citrulline + watermelon flesh	28.98 ± 3.24*	100.43 ± 6.13*	29.09 ± 5.47*	5
Watermelon rind				
Control	100	100	100	5
Oxytocin	135.17 ± 7.62*	152.74 ± 8.73*	156.50 ± 9.93*	5
Oxytocin + L-citrulline + watermelon rind	23.05 ± 1.39*	105.07 ± 6.92	22.10 ± 5.18*	5

The *P*-values for amplitude, frequency and AUC of 10 nM oxytocin treated are significantly different from the control (*$P < 0.05$). Mean ± S.E.M. are given; n is number of animals.

6.4.10 Effects of the Combinations of Watermelon Extracts and L-Citrulline on KCl-Induced Uterine Contraction

Watermelon Flesh Extract and L-Citrulline

As can be seen in Figure 6.14A, application of KCl (40 mM) to spontaneously contracting myometrium elicited an initial rapid phasic contraction followed by a sustained tonic contraction. Addition of the combination of the flesh extract (6 mg/mL) and L-citrulline (64 μM) to the myometrium in the continued presence of KCl produced a significant inhibition of the contraction (n = 5). This value was 40.59 ± 2.83% ($P < 0.05$) compared to the KCl control (100%).

Watermelon Rind Extract and L-Citrulline

As can be seen in Figure 6.14B, application of KCl (40 mM) to spontaneously contracting myometrium elicited an initial rapid phasic contraction followed by a sustained tonic contraction. Addition of the combination of the rind extract (5 mg/mL) and L-citrulline (64 μM) to the myometrium in the continued presence of KCl produced a significant inhibition of the contraction (n = 5). This value was 21.84 ± 2.64% ($P < 0.05$) compared to the KCl control (100%).

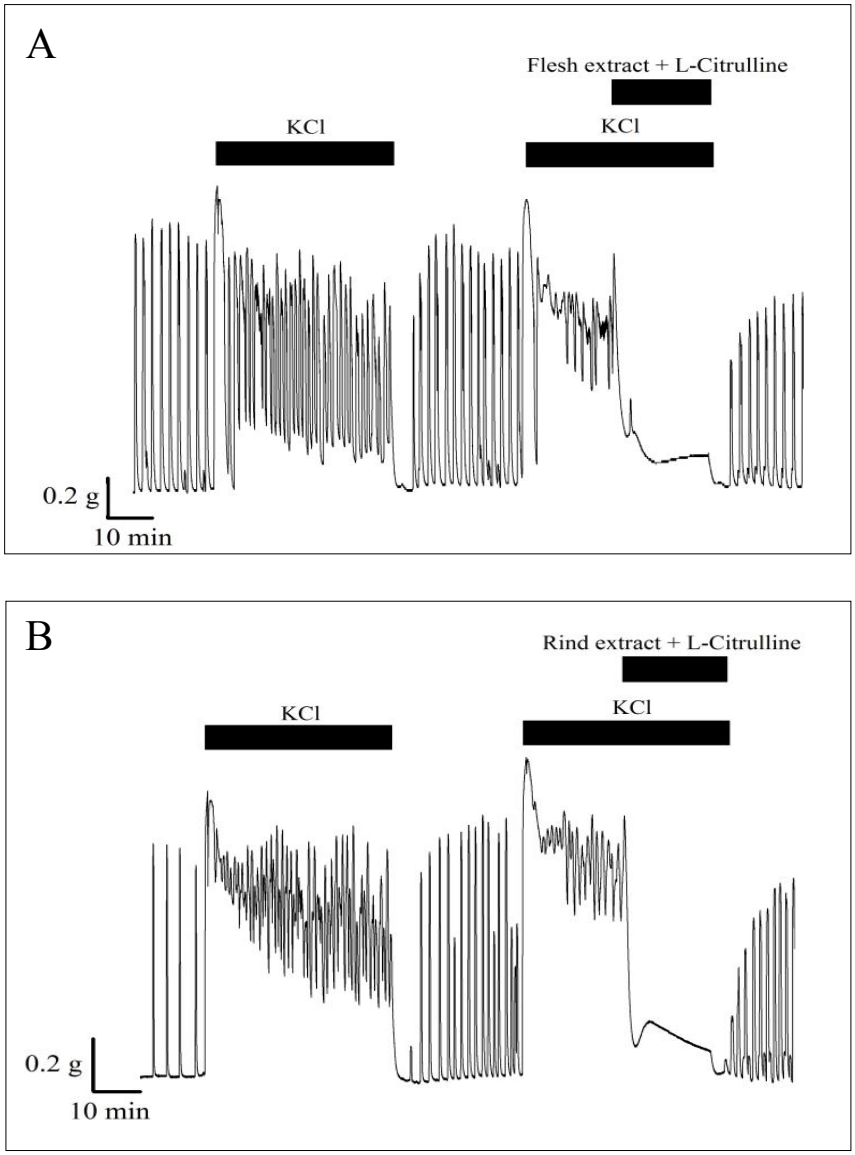

Figure 6.14 The effects of the combinations of watermelon extracts and L-citrulline on KCl-induced uterine contraction. The effects of the combination of watermelon flesh extract (6 mg/mL) and 64 μM L-citrulline (A) and the combination of watermelon rind extract (5 mg/mL) and 64 μM L-citrulline (B) on uterine contraction-induced by 40 mM KCl are shown (n = 5 for each).

6.5 Discussion

This study is the first to demonstrate the combination effects of watermelon extracts and L-citrulline on uterine contraction. The results revealed that the mechanisms whereby they exert their effects were dependent upon NO-cGMP-dependent pathway modulation. These findings suggest that the tocolytic effects of watermelon extracts may be due to L-citrulline. In addition, pre-incubation with TEA prevented the effects of watermelon extracts and L-citrulline exerting their effects, indicating that K_{Ca} channels are also served as the targets for their actions.

It is well established that sGC has been purified from a variety of tissues, including myometrium (Syal, Vedernikov, Chwalisz, Saade and Garfield, 1998). This protein can be activated by NO, leading to phosphorylation of PKG (Carvajal et al., 2000; Norman, 1996). This activation and subsequent phosphorylation of key proteins has been suggested as a mechanism of the modulation of smooth muscle tone by NO (Buhimschi et al., 1995; Carvajal et al., 2000; Yallampalli et al., 1994). It was reported that cyclic nucleotide can mediate smooth muscle relaxation by a variety of mechanism involving alterations of $[Ca^{2+}]_i$ or the sensitivity of the contractile apparatus to that cation (Carvajal et al., 2000; Lee, Li and Kitazawa, 1997; Wu, Somlyo and Somlyo, 1996). In the present study, watermelon extracts and L-citrulline caused the inhibitory effects in rat uterine contraction. By using L-NAME, the possible involvement of NO could be examined. The results showed that the inhibitory effects of watermelon extracts and L-citrulline were partially inhibited by L-NAME. These findings, therefore, indicate that the NO pathway modulation may be implicated with the action of the plant extracts and L-citrulline.

It is well known that NO is a potent relaxant of uterine smooth muscle with an action of mediated by cGMP (Buhimschi et al., 1995; Rizzo, Trisolini, Spedicato, Mutinati, Minoia and Sciorsci, 2011; Yallampalli et al., 1994). This current study proposed that LY 83583 also markedly reduced the inhibitory effects induced by watermelon extracts and L-citrulline. It is well established that cGMP activated by NO elicits the relaxation through PKG signaling (Carvajal et al., 2000; Lee et al., 1997; Wu et al., 1996). However, there is evidence that NO does not require the activation of cGMP to impede the contraction (Bradley, Buxton, Barber, Mcgaw and Bradley, 1998; Buxton, Kaiser, Malmquist and Tichenor, 2001; Kuenzli et al., 1998). Weiner, Knowles, Nelson and Stegink (1994) indicated that during pregnancy, increase of myometrial cGMP levels is independent of NO modulation. In addition, the increase in cGMP is at least one mechanism in responsible for suppressing the myometrial stretch reflex during pregnancy (Weiner et al., 1994). In contrast, in other studies revealed that cGMP can modulate myometrial contractile activity (Buhimschi et al., 1995; Demirkoprulu et al., 2005; Izumi and Garfield, 1995; Weiner et al., 1994; Yallampalli et al., 1994). Demirkoprulu et al. (2005) reported that at the same concentrations of YC-1, a NO-independent sGC activator, and DETA/NO, a NO donor, the contents of cGMP in myometrial strips pre-incubated with YC-1 were greater than myometrial strips pre-incubated with DETA/NO. In addition, DETA/NO was less inhibited the amplitude and the frequency of the contraction when compared with YC-1, implying that YC-1 may have multi-targets in responsible for the modulation of force. Inhibitory effects of YC-1 may be due to both the stimulation of sGC and K_{Ca} channels (Cetin, Kaya, Demirkoprulu, Karadas, Duran and Cetin, 2004; Demirkoprulu et al., 2005). It is demonstrated that medicinal plants are present as

numerous chemical constituents, which usually exert their effects through muti-targets and muti-pathways (Cao et al., 2008). Taken together, it is, therefore, thought that the watermelon extracts may exert their effects by two mechanisms (see below). The first mechanism is probably via an interaction with the sGC enzyme, causing an increase in cGMP and hence relaxation though a decrease in $[Ca^{2+}]_i$ (Carvajal et al., 2000). The second is by a direct interaction with other targets proteins to decrease in myofilament Ca^{2+} sensitivity (Bolz et al., 2003; Stuart-Smith, Warner and Jones, 1998). As can be seen, LY 83583 blocked the tocolytic effects-induced by watermelon extracts and L-citrulline; however, substantial relaxation remained. Thus, by using LY 83583, it is reasonable to speculate that the resultant inhibitory effects of watermelon and L-citrulline on rat myometrium could be due to the stimulation partially and/or completely sGC and cGMP may play a role in responsible for the tocolytic action. The content of cGMP is regulated by a balance between the ratio of synthesis by guanylate cyclase and the hydrolysis breakdown to GMP by cyclic nucleotide phosphodiesterase (PDE) enzymes (Carvajal et al., 2000; Wu et al., 1996). PDE5 is found in uterine smooth muscle cells and appears to play a significant role in regulating smooth muscle tone (Wu et al., 1996). Therefore, further work is necessary to examine the role of PDE in the effects of the plant extracts on rat uterine contractility and the potentiating of cGMP signaling.

The role of NO and cGMP in the hyperpolarization of the smooth muscle cell membrane remains controversial. However, there is evidence that NO can activate K_{Ca} channels directly (Bolotina et al., 1994). Moreover, some evidences suggested that NO was found to activate K_{Ca} channels independent of cGMP concentrations (Bradley et al., 1998; Norman, 1996). K^+ channels are important target proteins

related to the regulation of relaxation of uterine smooth muscle (Bradley et al., 1998; Buxton et al., 2001; Hoffmann, Stanke-Labesque, Fanchin, Dilaï, Pons and Ayoubi, 2003). It was demonstrated that K_{Ca} channels are the predominant type expressed in human (Khan, Smith, Morrison and Ashford, 1997) and rat (Anwer, Toro, Oberti, Stefani and Sanborn, 1992) myometrium. The opening of these channels results in an outward current of K^+ ions, implying the membrane hyperpolarization of smooth muscle cells, and subsequently suppresses Ca^{2+} entry through L-type Ca^{2+} channels (Anwer et al., 1992; Khan et al., 1997). To investigate the involvement of K_{Ca} channels in the tocolytic effects of watermelon extracts and L-citrulline, TEA (5 mM) was used. The results indicated that TEA can prevent the inhibitory effects of the plant extracts and L-citrulline on uterine contractility. Thus, the relaxant effects of watermelon extracts and L-citrulline seem to be due to the most selective effect on the K_{Ca} channels in uterine smooth muscle.

The combinations of watermelon extracts and L-citrulline produced an additive effect on both spontaneous and agonists-induced contractions. In addition, the effects of the combinations on uterine force were greater than that produced by their individual EC_{50} values. These findings indicate that the tocolytic effects of watermelon extracts may be due to L-citrulline. Moreover, the combinations of watermelon extracts and L-citrulline can interact additionally and therefore this drug association may represent a therapeutic advantage for the clinical treatment of various diseases (Williamson, 2001).

Medicinal plants showing NO production can be served as the tocolytic drug, which may have potential for the prevention and treatment of miscarriage and/or other disorders related to NO in reproductive function, including dysmenorrhea and preterm

labor (Norman, 1996). The results of this present study could be useful for further study to define the effects of watermelon on human myometrium *in vitro*.

In conclusion, this study investigated the tocolytic effects of watermelon extracts on isolated rat uterus and the possible mechanisms. The results indicated that the inhibitory effects of watermelon extracts and L-citrulline were via NO-cGMP-dependent pathway modulation. In addition, K_{Ca} channels may be the target of the plant extracts and L-citrulline to exert their tocolytic effects. Finally, watermelon extracts and L-citrulline can interact additionally to reduce the contractile force, indicating that the tocolytic effects of watermelon extracts may be due to L-citrulline.

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CHAPTER VII

CONCLUSION

Medicinal plants offer new sources of natural molecules that can be used to suppress myometrial contractile activity (tocolytic agents). Watermelon (*Citrullus lanatus*) is an interesting target for pharmaceutical investigation due to its specific amino acids, L-citrulline and L-arginine. These amino acids are associated with the production of nitric oxide (NO), a potent vasodilator. Ethnopharmacological relevance indicated that watermelon has antioxidant, cardioprotective, and anti-inflammatory properties. However, little is known about the tocolytic effects.

To the best of our knowledge, the inhibitory effects of watermelon extracts on rat uterine contraction have not yet been elucidated. Thus, this thesis was aimed; 1) to investigate the effects of the extracts from watermelon on rat uterine contractions; 2) to investigate the mechanisms whereby the extracts exerted their effects; and 3) to examine whether the effects of the extracts were due to their major constituents, L-citrulline and/or L-arginine. The major findings can be summarized as follows.

7.1 Dose Dependency of Watermelon Extracts

The inhibitory effects of watermelon flesh and rind extracts (2-8 mg/mL) were dose-dependent (Chapter III). The EC_{50} values of flesh and rind extracts were 6.13 ± 1.35 and 5.26 ± 1.08 mg/mL, respectively. Flesh extract at the dose of 6 mg/mL and rind extract at the dose of 5 mg/mL significantly decreased in the amplitude and the mean integral force but not the frequency of the contractions. The frequency of contractions increased when watermelon extracts were added. This may increase the intrinsic pacemaking mechanism in the uterus and shorten the action potential. However, the mechanism underlying of this effect is unclear. In addition, it was also found that the tocolytic potency of watermelon rind extract was greater than that produced by watermelon flesh extract. It has been demonstrated that rind contained more L-citrulline than flesh on dry weight basis. Therefore, this could be the reason why the tocolytic potency of the rind extract in this present study was greater than that produced by the flesh extract.

7.2 Effects of Watermelon Extracts on Agonists-Induced Uterine Contractions

Watermelon extracts depressed the contractions induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), oxytocin, and potassium chloride solution (KCl) (Chapter IV). They also reduced $PGF_{2\alpha}$- and oxytocin-induced uterine force in the absence of external Ca^{2+} and partially inhibited contraction induced by increasing external Ca^{2+}. In addition, the extracts produced a marked decrease in tonic contractions produced by oxytocin-induced uterine contraction in the presence of KCl. These findings indicated that the

tocolytic effects of watermelon extracts were via the inhibition of both Ca^{2+}-dependent and Ca^{2+}-independent pathways of force regulation.

7.3 Dose Dependency of L-Citrulline and L-Arginine

It has been reported that watermelon is rich in L-citrulline and L-arginine; the contents that play a crucial role in the production of the potent vasodilator, NO. Thus, it was of interest to investigate whether the tocolytic effects of the extracts may be due to L-citrulline or L-arginine. The results showed that L-citrulline and L-arginine (1×10^{-6} to 1×10^{-3} M) had a dose-dependent effect on spontaneous contraction (Chapter V). The EC_{50} values of L-citrulline and L-arginine were 64 ± 4.06 and 104.12 ± 2.03 μM, respectively. The tocolytic effect of L-citrulline was greater than that produced by L-arginine. This might be due to the saturation of the substrate, L-arginine, for NO synthase. It was suggested that the L-citrulline to L-arginine recycling pathway is localized to caveolae and may serve as the principle source of available L-arginine.

7.4 Effects of L-Citrulline and L-Arginine on Agonists-Induced Uterine Contractions

L-citrulline and L-arginine suppressed the contractions induced by $PGF_{2\alpha}$, oxytocin, and KCl. They also reduced $PGF_{2\alpha}$- and oxytocin-induced uterine force in the absence of external calcium and partially inhibited contraction induced by increasing external Ca^{2+}. In addition, L-citrulline and L-arginine caused a marked decrease in tonic contractions produced by oxytocin-induced uterine contraction in the

presence of KCl. These data revealed that the tocolytic effects of L-citrulline and L-arginine were via the inhibition of both Ca^{2+}-dependent and Ca^{2+}-independent pathways of force regulation and that their effects were similarly to those of watermelon extracts.

7.5 Effects of Watermelon Extracts and L-Citrulline on Nitric Oxide

There is evidence that both fruit flesh and rind of watermelon are the excellent sources of in L-citrulline. In addition, L-citrulline produced the relaxant effects on rabbit vascular smooth muscle and rat aortic rings mainly through the NO-cGMP pathway modulation. Thus, it was of interest to investigate whether the tocolytic effects of the extracts and L-citrulline were via NO-cGMP pathway. The results exhibited that the tocolytic effects of watermelon extracts and L-citrulline were via the production of NO and cGMP. Moreover, TEA partially reversed the inhibitory effects of the plant extracts and L-citrulline, suggesting that calcium-activated potassium channels may be involved in the mechanism of action of watermelon extracts and L-citrulline (Chapter VI).

The combination of the extracts and L-citrulline elicited an additive effect on spontaneous contraction and the contractions induced by $PGF_{2\alpha}$, oxytocin, and KCl. These findings suggest that the tocolytic effects of watermelon extracts may be due to L-citrulline.

Based on the results of present study, watermelon extracts, L-citrulline, and L-arginine produced a significant suppressive effect on oxytocin-induced contraction, suggesting that they may be effective in the prevention of preterm labor. In addition,

watermelon extracts, L-citrulline, and L-arginine exhibited the inhibitory effects on $PGF_{2\alpha}$-induced contraction suggested that they may also be effective in the treatment of primary dysmenorrhea.

In conclusion, the present data clearly showed that watermelon has a potent tocolytic effect on uterine contraction. The mechanisms of action were via Ca^{2+}-dependent and Ca^{2+}-independent regulation of smooth muscle contraction pathways as well as via NO-cGMP pathway modulation. The mechanisms of action can be shown in Figure 7.1. However, the experiments were undertaken in an animal model *in vitro*. It would be, therefore, interesting to further investigate such the effects in a human model *in vitro*.

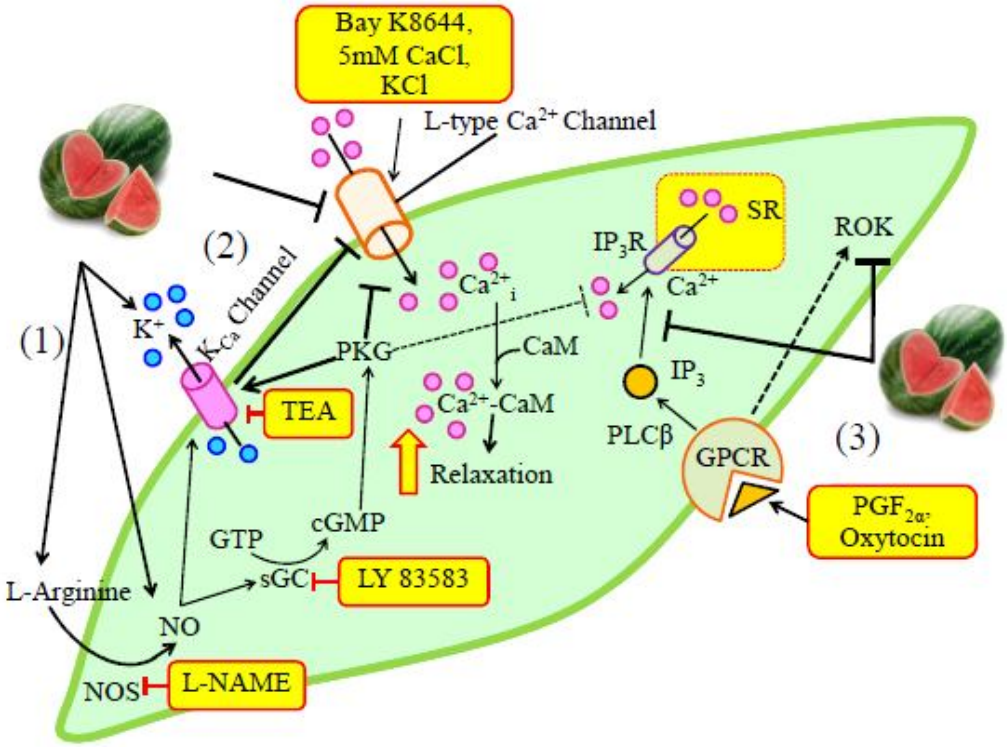

Figure 7.1 Schematic representation of the mechanisms underlying of the effects of watermelon extracts (WMEs) on rat uterine contraction. Ca^{2+} enters cells through L-type Ca^{2+} channels. MLCK is activated by four Ca^{2+} ions bound to CaM, leading to myosin phosphorylation and subsequent cross-bridge cycling. **(1)** WMEs may serve as a natural nitric oxide (NO) donor or may activate NO synthase (NOS) directly to increase the production of NO in uterine smooth muscle cells which was blocked by L-NAME, a non-selective NOS inhibitor. NO activates soluble guanylate cyclase (sGC) to produce cGMP which was blocked by LY 83583, a soluble guanylate cyclase inhibitor. **(2)** WMEs itself or NO produced by WMEs stimulates calcium-activated potassium (K_{Ca}) channels which were blocked by TEA, a K_{Ca} inhibitor. WMEs itself or protein kinase G (PKG) inhibits voltage-gated Ca^{2+} channels (VGCC) which was partially reversed by Bay K8644, a specific L-type Ca^{2+} channel activator,

or high Ca^{2+}. **(3)** WMEs may interrupt the $G\alpha_{q/11}$-PLCβ-IP_3 pathway mediated by $PGF_{2\alpha}$ or oxytocin and may hamper the ROK pathway. **Abbreviations**: calcium-calmodulin complex (Ca-CaM), protein kinase G (PKG), cyclic guanosine monophosphate (cGMP), soluble guanylyl cyclase (sGC), phospholypase Cβ (PLCβ), phosphatidylinositol 4,5-bisphosphate (PIP_2), 1,2-diacylglycerol (DAG), inositol 1,4,5-trisphosphate (IP_3), calcium-activated potassium channel (K_{Ca} channel), nitric oxide synthase (NOS), tetaethylamonium chloride (TEA), rho-associated kinase (ROK).

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